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Development and evaluation of new therapeutic interventions for chronic hepatitis C virus infection

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DEVELOPMENT AND EVALUATION OF NEW THERAPEUTIC INTERVENTIONS FOR HEPATITIS C VIRUS INFECTION

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To my family

ABSTRACT

The hepatitis C virus (HCV) is a major global health problem with around 130-150 million individuals chronically infected and it is estimated that 2 millions are newly infected each year. The HCV infection is one of the major causes of liver disease and the infection is characterized by a slow and silent progression. Patients infected with HCV have an increased risk of developing fibrosis, cirrhosis and hepatocellular carcinoma.

The effectiveness of new treatments has drastically improved during the last years and the cure rate is today around 90 %. However, several obstacles remain to be solved. Firstly, the treatment is associated with high costs. Secondly, there are still uncertainties whether patient groups such as children, pregnant women and treatment-experienced patients will be eligible for this treatment. Finally, the direct-acting antiviral (DAA) treatment does not protect against a re-infection. Also, only around 10 % of all chronic HCV carriers have access to treatment and the highest prevalence of HCV are seen in developing countries, which highlights the need of alternative less-expensive treatment strategies.

In this thesis we developed and characterized new treatments strategies for HCV based on genetic vaccines and re-direction of T cells. We utilized the non-structural (NS) 3/4A and 5A proteins of HCV as vaccine antigens and as targets for re-directed T cells. Both the NS3/4A and NS5A proteins have essential functions in HCV life cycle.

We generated an NS5A-based DNA vaccine and performed detailed characterization of its *in vivo* immunogenicity. We have previously developed a DNA vaccine based on NS3/4A that effectively primes immune responses *in vivo*. A codon optimized NS5A vaccine sequence was delivered intramuscularly in combination with *in vivo* electroporation for efficient uptake. The DNA vaccine activated NS5A-specific immune responses in both wild type and NS5A-transgenic (Tg) mice, with dysfunctional HCV-specific T cells. The vaccine primed responses were functional *in vivo* as evidenced by protection against *in vivo* tumor growth of NS5A-expressing cells. Furthermore, the vaccines were also used to develop NS3- and NS5A-specific T cell receptors (TCRs). Both the NS3/4A and NS5A TCRs were able to inhibit HCV RNA replication *in vitro* albeit using different mechanisms, possibly related to differences in the T cell avidity. The NS3/4A and the NS5A vaccines activated distinct HCV-specific immune responses. The NS3/4A vaccine was dependent on IFNγ and $CD8^+$ T cells but not $CD4^+$ T cells, whereas the NS5A vaccine was dependent on both $CD4^+$ and $CD8^+$ T cells but not on IFNγ. This is consistent with high-avidity NS3-specific TCRs inducing polyfunctional T cells, as compared to the low-avidity NS5A TCRs inducing monofunctional T cells. Finally, we analyzed the possibility to improve the intrinsic immunogenicity of the NS5A vaccine. We have previously showed that the immunogenicity of a NS3/4A-based vaccine could be significantly increased by co-expression with IL-12 and the addition of heterologous sequences from hepatitis B virus core antigen (HBcAg). However, the immunogenicity of NS5A was enhanced by addition of HBcAg sequences, but not co-expression of IL-12.

In conclusion, we have developed and evaluated new potential therapeutic interventions for the treatment of chronic HCV infection. Although the effective DAA treatment is available, it is clear that alternative treatment strategies are needed. These should be associated with lower costs and preferentially induce a post cure immunity that protect against re-infection.

LIST OF PUBLICATIONS

I. **Fredrik Holmström**, Anna Pasetto, Veronica Nähr, Anette Brass, Malte Kriegs, Eberhard Hildt, Kate E. Broderick, Margaret Chen, Gustaf Ahlén, and Lars Frelin. *A synthetic codon-optimized hepatitis C virus nonstructural 5A DNA vaccine primes polyfunctional $CD8^+$ T cell responses in wild-type and NS5A-transgenic mice.* **J Immunol**. 2013 Feb 1;190(3):1113-24.

II. Anna Pasetto, Lars Frelin, Soo Aleman, **Fredrik Holmström**, Anette Brass, Gustaf Ahlén, Erwin D. Brenndörfer, Volker Lohmann, Ralph Bartenschlager, Matti Sällberg, Antonio Bertoletti, and Margaret Chen. *TCR-redirected human T cells inhibit hepatitis C virus replication: hepatotoxic potential is linked to antigen specificity and functional avidity.* **J Immunol**. 2012 Nov 1;189(9):4510-9.

III. Antony Chen, Gustaf Ahlén Erwin D. Brenndörfer, Anette Brass, **Fredrik Holmström**, Margaret Chen, Jonas Söderholm, David R. Milich, Lars Frelin, and Matti Sällberg. *Heterologous T cells can help restore function in dysfunctional hepatitis C virus nonstructural 3/4A-specific T cells during therapeutic vaccination.* **J Immunol**. 2011 May 1;186(9):5107-18.

IV. **Fredrik Holmström**, Margaret Chen, Anangi Balasiddaiah, Gustaf Ahlén, and Lars Frelin. *Functional differences in hepatitis C virus nonstructural (NS) 3/4A- and 5A-specific T cell responses.* **Submitted manuscript**.

RELATED PUBLICATIONS

I. Anne Fournillier, Lars Frelin, Emilie Jacquier, Gustaf Ahlén, Anette Brass, Estelle Gerossier, **Fredrik Holmström**, Kate E. Broderick, Niranjan Y. Sardesai, Jean-Yves Bonnefoy, Geneviève Inchauspé, and Matti Sällberg. *A heterologous prime/boost vaccination strategy enhances the immunogenicity of therapeutic vaccines for hepatitis C virus*. **J Infect Dis**. 2013 Sep;208(6):1008-19.

II. Gustaf Ahlén, **Fredrik Holmström**, Anna Gibbs, Mats Alheim, and Lars Frelin. *Long-term functional duration of immune responses to HCV NS3/4A induced by DNA vaccination.* **Gene Ther**. 2014 Aug;21(8):739-50.

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LIST OF ABBREVIATIONS

aa	amino acid
ALT	alanine aminotransferase
APC	antigen presenting cell
co	codon optimized
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DNA	deoxyribonucleic acid
ER	endoplasmatic reticulum
HBcAg	hepatitis B core antigen
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HLA	human leucocyte antigen
i.m.	intramuscular
IFN	interferon
IL	interleukin
IRF	interferon regulatory factor
MHC	major histocompatibility complex
NK	natural killer cell
NS	non-structural
ORF	open reading frame
pDNA	plasmid DNA
RBV	ribavirin
RdRp	RNA dependent RNA polymerase
RNA	Ribonucleic acid
TCR	T cell receptor
Tg	Transgenic
Th	T helper
TLR	toll like receptor
TNF	tumor necrotis factor
UTR	untranslated region

1 INTRODUCTION TO HEPATITIS

The liver is a vital organ for humans, which is located in the upper right part of the abdomen. Several important functions as protein syntheses, production of compounds necessary for digestion, detoxification and many others are carried out by the liver.

The cause of hepatitis, which means inflammation (*greek itis*) of the liver (*greek hepar*), are several factors such as viral infection, bacterial infection, metabolic disorders, alcohol, toxins drugs, chemicals, etc. Until now five viruses have been identified to infect the liver, hepatitis A, B, C, D and E. There are also other viruses that may infect the liver such as Epstein Barr virus (EBV), Cytomegalovirus (CMV), Yellow fever, Ebola, Dengue and Adenoviruses.

The primary site of hepatitis virus replication is the hepatocytes that occupy approximately 80% of the liver volume. The entry of the liver is via the blood stream, which gives the virus access to the hepatocytes and can cause an acute infection. The hepatitis infection can be asymptomatic or symptomatic with common symptoms such as jaundice or yellowish skin (bilirubin in the blood), fatigue, nausea, myalgia, stomach pain, vomiting and fever. The acute infection of hepatitis often results in measurable elevated levels of liver enzymes in the blood. A chronic infection is a persistent viral infection for more than six months and this chronic phase proceeds as either asymptomatic or symptomatic where the symptomatic often is associated with active inflammation and increased risk of cirrhosis and hepatocellular carcinoma (HCC, liver cancer). Although all five known hepatitis viruses infect the liver they differ in genome, structure and also transmission routes.

1.1 HEPATITIS A VIRUS

The hepatitis A virus (HAV) was discovered in 1973 and belongs to the *Picornaviridae* virus family (1). It is a single stranded RNA virus transmitted via the fecal-oral route and causes only acute infection. The incubation time is 2-6 weeks and the infection is often symptomatic. There are both passive immunization (immunoglobulins) and prophylactic vaccination available (2).

1.2 HEPATITIS B VIRUS

The hepatitis B virus (HBV) was discovered in 1965 and belongs to the *Hepadnaviridae* virus family (3). It is a partially double stranded DNA virus transmitted via contaminated blood, sexual or vertical transmission. The hepatitis B virus has evolved a strategy of causing tolerance in infants during the neonatal development. The HBeAg, which is not required for replication, assembly or infection, is secreted and can pass through the placenta, enter the blood stream of the fetus and induce tolerance. During the delivery the infant gets infected and due to the already tolerized immune system 90 % of the infants develop chronic hepatitis compared to less than 10 % of the adults (4). It is estimated that a third of the world's population has been exposed to HBV and that approximately 350 million individuals are chronically infected. Chronic HBV is treated with interferon-α or antivirals such as entecavir and tenefovir (5). A prophylactic vaccine with effective protection is available (6)

1.3 HEPATITIS C VIRUS

The hepatitis C virus (HCV) was discovered 1989 and belongs to the *flaviviridae* virus family (7). It is a single stranded RNA virus transmitted mainly via contaminated blood. The infection leads into a

chronic infection in around 80 % of the acute cases and approximately 130-150 million infected people worldwide. Hepatitis C virus can also cause chronic infection and will be discussed in grater detail later.

1.4 HEPATITIS D VIRUS

The hepatitis D virus (HDV) or "deltavirus" was discovered in 1977 and belongs to the genus Deltavirus (8). It is a single stranded RNA virus lacking polymerase and envelope protein. Hepatitis D virus is therefore dependent of a human RNA-polymerase to replicate and also the HBV surface antigen to generate infectious viral particles. Hepatitis D virus is transmitted either as a co-infection with HBV or as a superinfection where the person is already chronically infected with HBV and at a later stage gets infected with HDV. A co-infection or superinfection is associated with more severe infection than HBV alone (4). Treatment for HDV are the same as for HBV.

1.5 HEPATITIS E VIRUS

The hepatitis E virus (HEV) was discovered 1983 and belongs to the *Hepeviridae* virus family (9). It is a single stranded RNA virus transmitted via the fecal-oral route and causes only acute infection. The incubation time is 3-8 weeks and the infection is often symptomatic. There is no specific treatment or vaccine available for HEV.

2 HEPATITIS C VIRUS

2.1 HISTORY

In the 1970s serological testing for hepatitis A and hepatitis B virus were developed and it was revealed that an not yet discovered virus causing hepatitis were existing, non-A and non-B hepatitis. In 1989 the genome of non-A and non-B hepatitis were cloned and characterized after experimental infection of chimpanzee and at the same time a serological test were developed (7, 10). The virus was named hepatitis C virus (HCV) and were similar in structure to flaviviruses (e.g. Dengue fever, West-Nile virus, Yellow fever virus) and pestiviruses (e.g. bovine viral diarrhea virus) in the Flaviviridae family and was therefore classified in the same family in the genus of Hepacivirus (11). Since the early 1990s, all blood used in medical centers are screened for HCV. In high-income countries, the transmission is almost exclusively occurring in intra venous drug users (12).

2.2 EPIDEMIOLOGY

The world's health organization (WHO) estimated that the worldwide prevalence of chronic HCV infection is around 130-150 million or approximately 2% of the world population (13). The country with the highest prevalence of HCV is Egypt with 10-20% of the population infected due to unsterile injection equipment during treatment of the general population against the parasite schistosomiasis in the 1980s (14-16). Prevalence of HCV in Europe is around 8 million people (2-3,5%) and in Sweden around 40,000 (0,5%) (17-19).

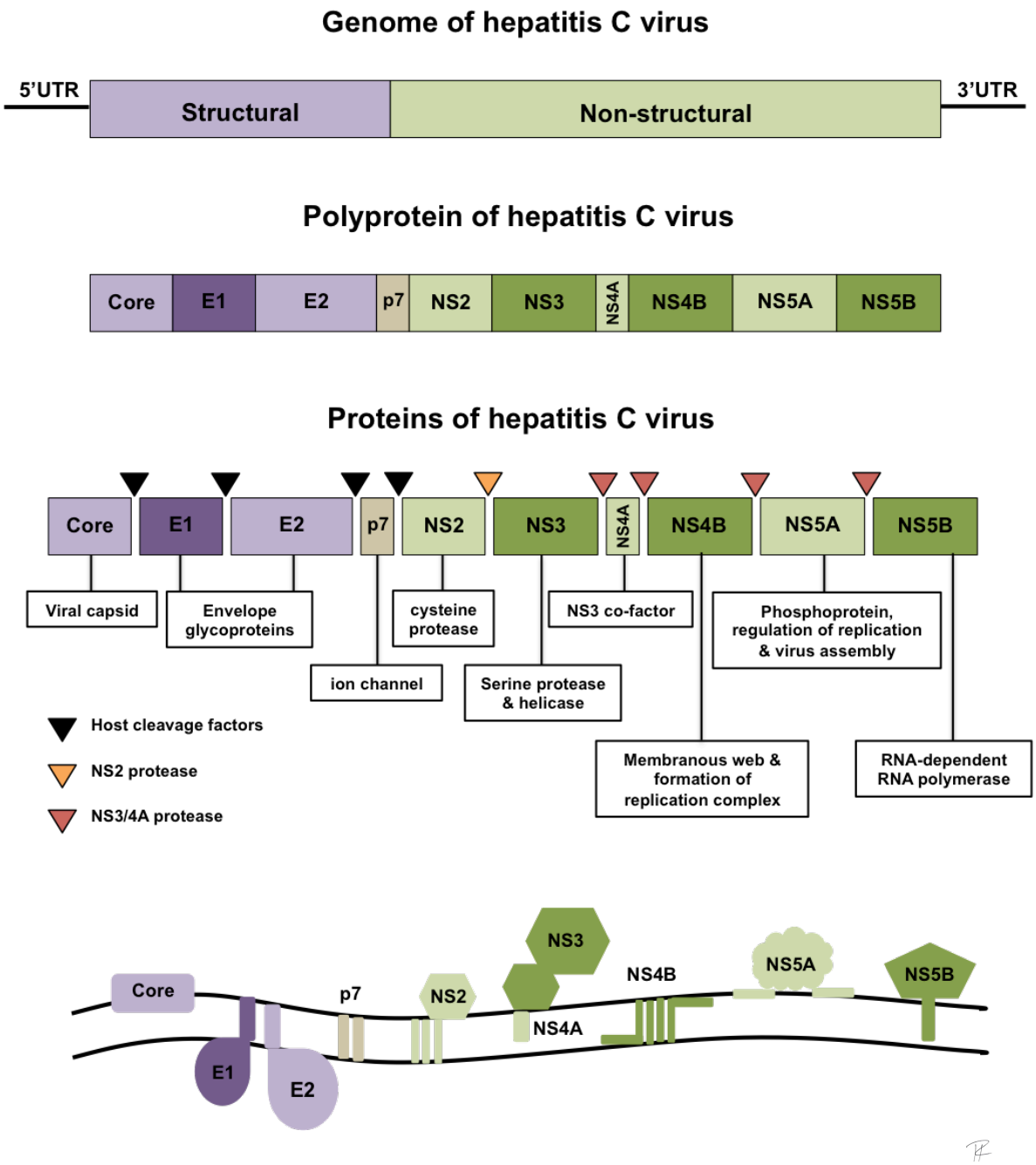

Figure 1. Schematic drawing of the hepatitis C virus genome and proteins.

2.3 HCV GENOME

The hepatitis C virus is consisting of a single stranded positive sense RNA molecule encapsulated in a spherical particle of 55-65 nm in diameter (20). The viral RNA genome is around 9,6 kb and encodes for one single open reading frame (ORF) that are translated into one polyprotein of approximately 3000 amino acids (7). Untranslated regions (UTR) in the 5´and 3´ ends of the genome contain essential secondary RNA structures for the viral replication. The 5´ UTR includes an internal ribosome entry site that initiates the translation of the polyprotein, which is then processed by host and viral proteases to individual structural proteins (core, envelope 1 and 2) and non-structural (NS) proteins (p7, NS3, NS4A, NS4B, NS5A and NS5B) (**Figure 1**). The core (c) protein is building up the nuclear capsid and is involved in regulation of functions in translation, RNA replication and assembly

of virus particles (21). The envelope (E) protein E1 and E2 are glycoproteins that are essential for receptor binding and receptor mediated endocytosis (22, 23). The p7 protein is an integral membrane protein with the activity of an ion-channel that it is required for formation of infectious virions (24, 25). The NS2 protein catalyzes the cleavage between NS2-NS3 in the polyprotein (26, 27) and NS3 are responsible for the cleavage of the polyprotein down stream of NS3 to the individual proteins. The NS3 protein has beside the function as a serine-protease (N-terminal domain, 180 aa) also a helicase/NTPase function (C-terminal domain, 442 aa), binding and unwinding the secondary structures of the viral RNA (28, 29). The NS3s function is dependent on the cofactor NS4A and its membrane-anchoring domain to be localized at the ER membrane and also to stabilize the protease from proteolytic degradation (30-32). The NS3 protein is also known for inhibiting the host cell innate immune response against HCV, discussed in "*Immune response and immune evasion in HCV infection"*. The NS4B protein is crucial for the RNA replication and formation of the membranous web at the ER membrane during viral replication (33-35). The NS5A is a multifunctional phosphoprotein with still not fully understood function/s. It is anchored to the ER membrane, essential for viral RNA replication, important for assembly of viral particles and interacts with several host and viral proteins (36-38). The NS5A protein is also involved in modifying the immune response to the infected cell, discussed in section: "*Immune response and immune evasion in HCV infection"*. The NS5B is the RNA-dependent RNA polymerase (RdRp) associated to the ER membrane and is the component in the replication complex catalyzing the process of duplicating the viral RNA (39, 40).

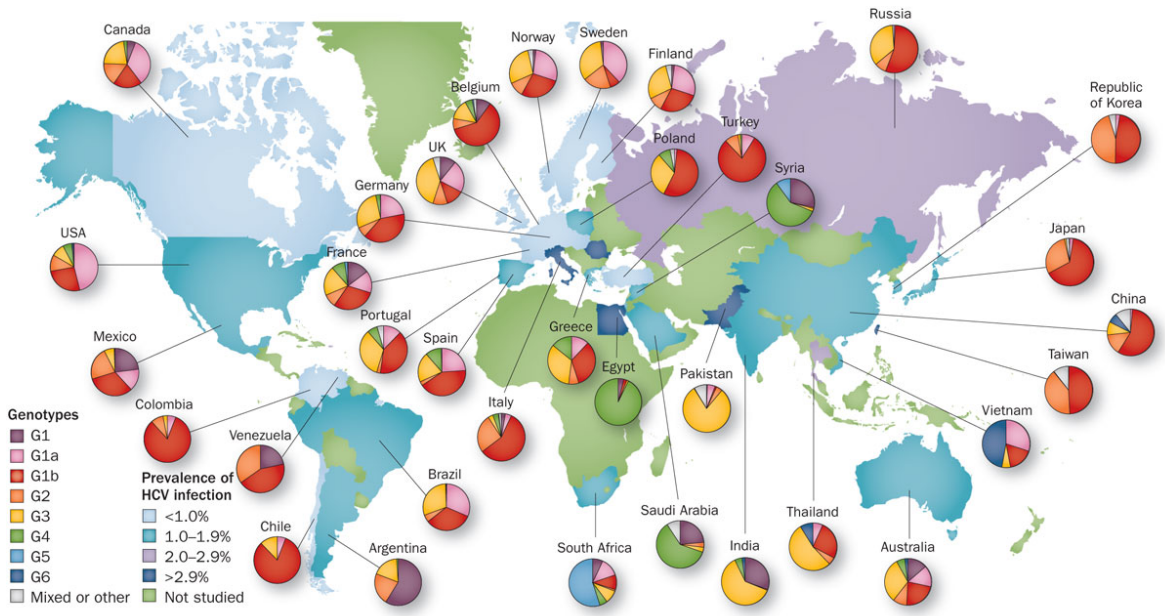

Figure 2. The estimated prevalence of HCV infection and the distribution of HCV genotypes across the world. Adapted by permission from Macmillan Publishers Ltd: [Nat Rev Gastroenterol Hepatol.] (41), copyright (2013).

2.4 GENOME DIVERSITY

The genome of hepatitis C virus is highly variable due to fact that the RNA-dependent RNA polymerase lack proof-reading activity. The high HCV replication rate of 10^{10}-10^{13} new virus particles per day result in a mutation rate of 10^{3} nucleotide substitutions per nucleotide and year. The high variability in the genome has resulted in 7 different genotypes (1-7) and many subtypes (a, b, c, etc.)

(**Figure 2**) (41). Genotype 1 represents over 50 % of all HCV infections and is distributed worldwide. Also genotype 2 is found worldwide. Genotype 3 are most common in southern Asia but also distributed worldwide. Genotype 4 is associated to northern Africa, genotype 5 in South Africa and genotype 6 in Asia. Genotype 7 has recently been identified in an emigrant from central Africa (42). The genotypes differ from each other with 30-35 % and the subtypes can vary between 20-25 % (43). Due to the mutation rate of HCV there is also variations within the viral genome of the same patient. This is referred as quasispecies and can vary between 1-9 %. There are regions of the HCV genome that are less variable such as the 5´UTR with nucleotide sequences more than 90 % homology among genotypes. Other regions with low variability are the core, NS3 and parts of NS5 (44). The segment with the highest variable sequence is the hyper variable region in the E2 protein with as much as 50 % diversity among genotypes.

2.5 VIRAL LIFE CYCLE

The hepatitis C virus only infects humans and chimpanzees where the hepatocytes are the primary target cell for propagation but there have been reports that also dendritic cells, B cells and other cells can be infected but it is debated whether these cells really can propagate the virus.

2.5.1 Binding and entry

It has been shown that HCV is associated with low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) when circulating in the blood. The first receptors that mediate the entry are the LDL receptor and glycosaminoglycans (GAG) but the exact role still remains to be determined (45, 46). The E2 protein then binds CD81 (tetraspanin) and scavenger receptor B-1 (SR-B1) followed by interaction with the tight junction proteins claudin-1 (CLDN1) and occluding (OCLN) to enter the target cell (47-50). Other receptors necessary for entry is cholesterol absorption receptor Niemann-Pick C1-like 1 (NP1L1), epidermal growth factor receptor (EGFR) and epherin receptor type A2 (51, 52). It has been shown that it is possible to make mouse hepatocytes susceptible to HCV entry by making the mice transgenic for the human CD81 and OCLN (50). After receptor binding, fusion of the viral envelope and the cell membrane takes place followed by endocytosis mediated by the protein claterin (53). The viral genome is then released into the cytosol by fusion of the endosomal membrane and viral envelope (**Figure 3**) (54).

2.5.2 Translation and processing of protein

The released single stranded RNA with positive sense is translated via the internal ribosome entry site (IRES) which means that the translation is a cap-independent translation mediated by the IRES (55). The translated polyprotein is then processed by host proteases and the viral NS2/3 protease (**Figure 3**) (56).

2.5.3 RNA replication

The replication occurs in a structure called the membranous web. This is a structure that is derived from the ER membrane and the NS4B protein has a central role in the formation of the membranous web (33). The RNA-dependent RNA polymerase (RdRp) or NS5B is the essential protein and the catalytic component of the replication complex. The NS3 protein with helicase function unwinds the secondary structures on the viral RNA and the NS5B protein is synthesizing the negative single stranded RNA out of the positive stranded RNA genome (28, 29). The synthesized RNA strand with

negative sense is then used as a template for generation of new viral RNA genome with positive sense (**Figure 3**).

2.5.4 Assembly and release

It has recently been shown that the assembly of the viral proteins and RNA occurs in the ER membrane by both viral and host factors (57). The particle formation where core units are building up the nucleocapsid incorporating viral RNA is arranged in co-localization with lipid droplets (58, 59). The formed virus particles are then surrounded by membrane from the ER, in which heterodimers of the glycoprotein E1 and E2 are embedded. The virus particle is then released in a secretory pathway from the cell (**Figure 3**).

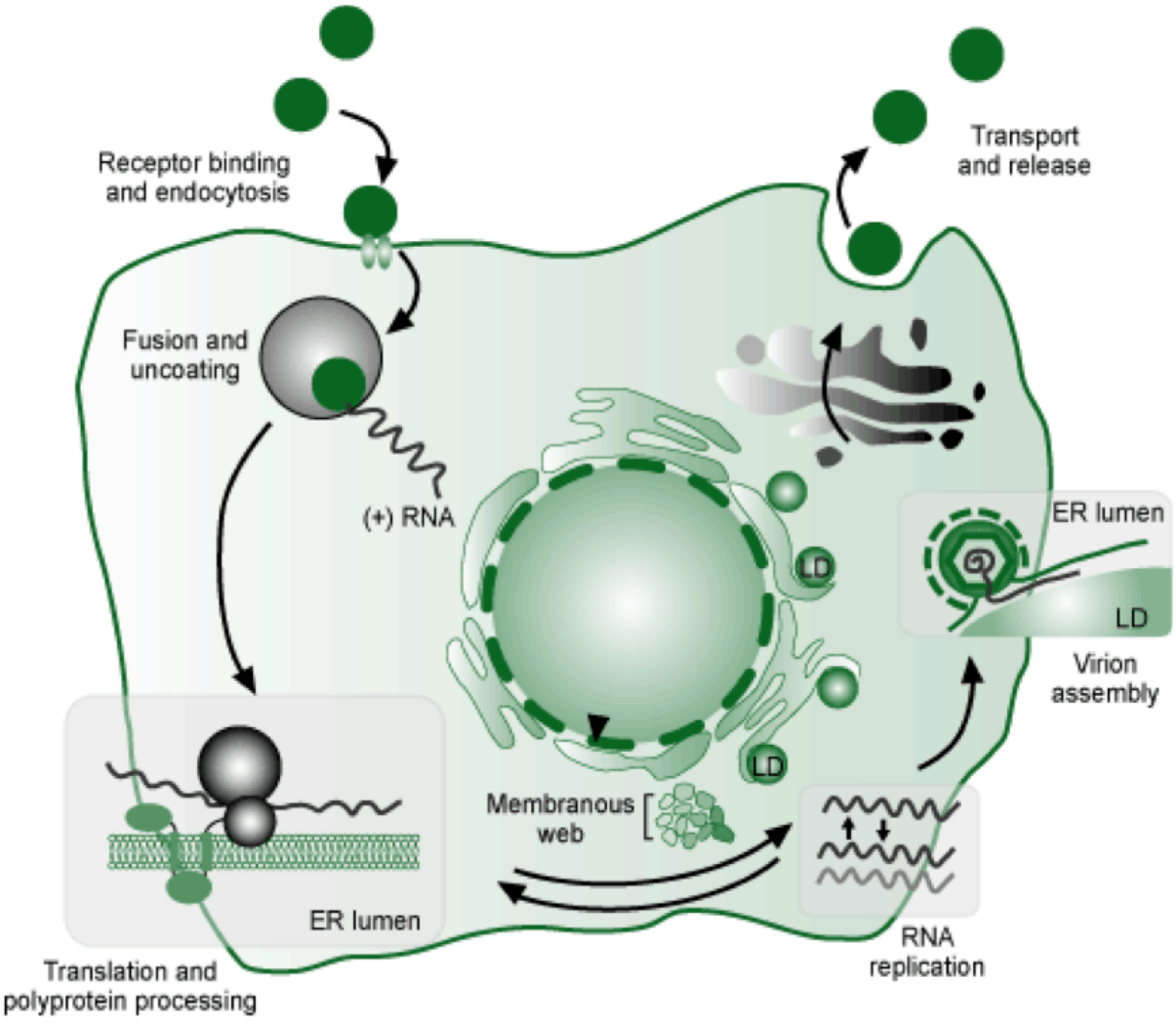

Figure 3. Illustration of the HCV life cycle. Adapted with permission from (60).

2.6 HCV INFECTION

Hepatitis C virus is a blood-borne virus where the main transmission route differs between high-income and low-income countries. In high-income countries the main transmission route is via contaminated needles and syringes in the group of intra venous drug users. In low-income countries it is instead nosocomial transmission that is the main rout of transmission such as blood transfusions and

transplantations. Sexually and vertical (mother to child) transmission of HCV is rare but still a risk of transmission as well as body piercing and tattooing. In approximately 80 % of the cases the HCV infection turns chronic meaning that the infection of the liver has been persistent over a period of six months. Spontaneous resolution of the infection occurs in approximately 20 % of the cases often without any or with mild symptoms (61). The chronic infection is characterized by elevated levels of alanine aminotransferase (ALT), anti-HCV antibodies and detectable viral RNA. The inflammation of the liver progresses gradually to fibrosis and after 10-30 years of chronic HCV infection around 20 % of the patients will develop cirrhosis with an increased risk of developing hepatocellular carcinoma (**Figure 4**). The acute infection is difficult to recognize because there are no or non-specific symptoms. Thus, acute HCV infection is only treated if the individual is confirmed HCV positive with known time point for exposure, for example accidental needle stick. If the patient is treated with IFNα monotherapy during the acute phase most resolve the infection (62, 63). Resent development of direct acting antiviral drugs have revolutionized the treatment of chronic HCV infection with cure rates over 90 %, discussed in more detail under *"Treatment"*.

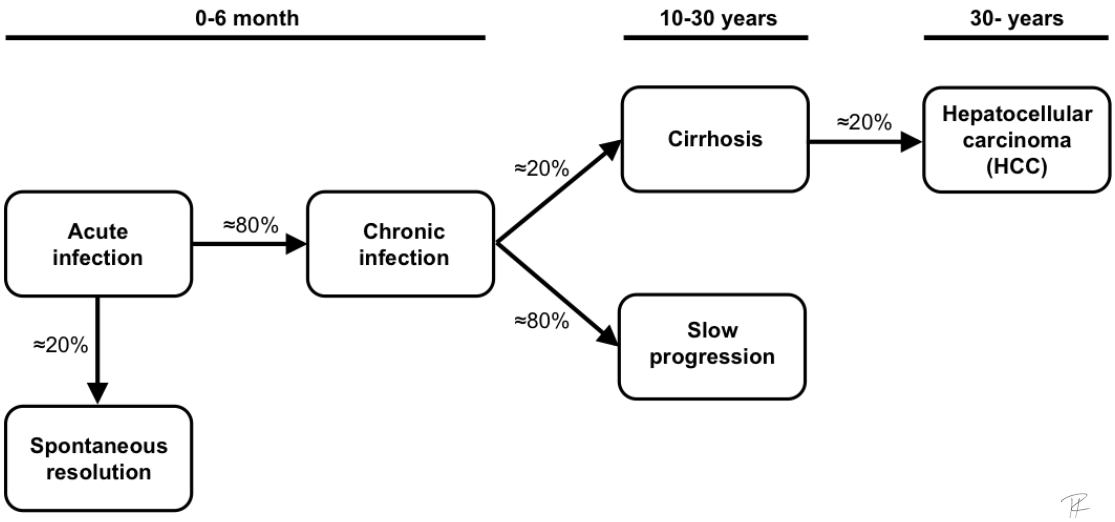

Figure 4. Flow chart over of the clinical course of HCV infection.

2.7 IMMUNE RESPONSE AND IMMUNE EVASION IN HCV INFECTION

The immune system is divided into two separate parts, innate and adaptive. The first line of defense towards pathogens is the anatomical barrier in the case of HCV it is skin, mucous membrane and eyes that needs to be penetrated or passed through to get in contact with the blood flow and continue to the liver. In the liver, the innate immune system reacts with Kupffer cells (liver macrophages), natural killer cells (NK), natural killer T cells (NKT) and IFN response from hepatocytes and other cells. The adaptive immune system is activated later and consists of $CD4^+$ and $CD8^+$ T cells and B cells. The inherent tolerogenicity of liver and its role in regulating innate and adaptive immunity may cause weak or no responses to invading pathogens instead of priming a strong immune response to the pathogen. In addition, HCV has evolved different strategies to avoid the host immune response, which will be discussed below.

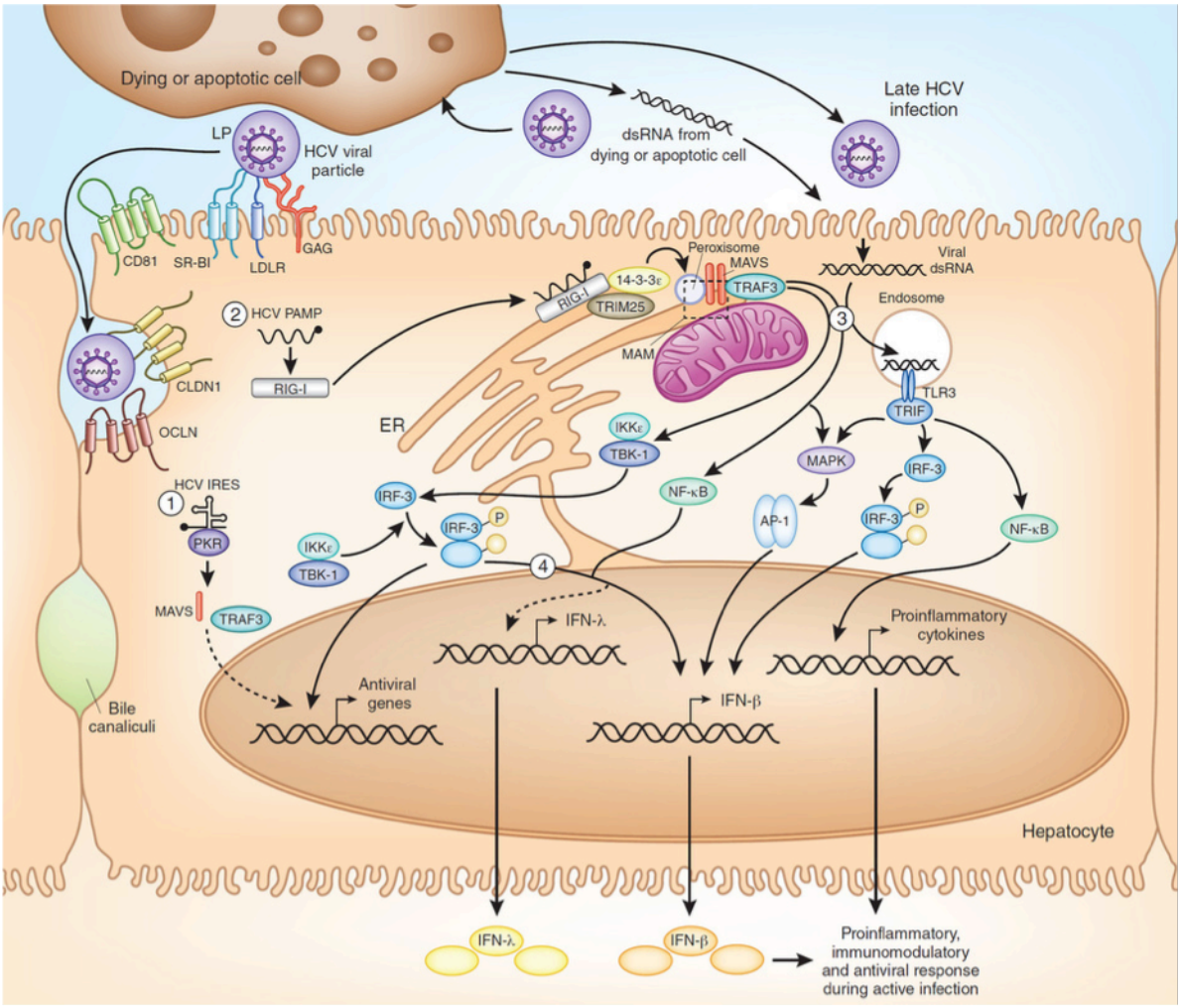

Figure 5. Illustration of the innate immune activation during HCV infection. Adapted by permission from Macmillan Publishers Ltd: [Nat Med.] (64), copyright (2013).

2.7.1 Innate immune response

2.7.1.1 Intracellular immune activation upon virus recognition

Pattern recognition receptors (PRR) like toll like receptors (TLR) and retinoic acid-inducible gene (RIG)-I are important in the response against virus infection by recognition of pathogen-associated molecular patterns (PAMPs). TLR-3, protein-kinas R (PKR) and RIG-I are all present in hepatocytes and involved in sensing the viral replication. Upon HCV infection the double stranded RNA as a result of the viral replication is recognized by these receptor. The activation of PKR, RIG-I and TLR-3 leads to a signaling cascade (PKR and RIG-I via mitochondrial antiviral signaling protein (MAVS) and TLR-3 via TIR domain-containing adapter inducing IFNβ (TRIF)) with induction of interferon regulatory factor (IRF)-3, NF-κB and production of type I and III IFNs (IFNα, β and λ), proinflammatory cytokines and chemokines, antiviral genes and apoptotic signal (64). This affects the infected cell and also the neighboring cells via the IFN signaling that induces the JAK/STAT pathway which leads to induction of a antiviral state and limits possibility for the virus to spread from cell-to-cell (**Figure 5**) (65). In the chronic infection these signaling pathways are blocked or altered by HCV using different strategies. The HCV NS3/4A protein is involved in interrupting the RIG-I signaling by

cleavage of MAVS. This block down stream signaling pathways and induction of NF-κB and IRF-3, which causes a lack of production or secretion of INFβ or antiviral genes (66, 67). The HCV protein NS5A are also involved in disturbing the innate immune system of HCV by interfering with PKR. The NS5A protein is inhibiting the PKR and the functions that PKR has upon sensing double stranded RNA. These functions are important for the innate immune system to inhibit translation, induction of apoptosis, type I interferons and antiviral genes. This evasion of the antiviral response has been described as an IFN sensitivity determining region (ISDR) dependent way of inhibition. The NS5A protein can also inhibit antiviral activity of IFN through interaction with 2'5' oligoadenylate synthetize (OAS) and by that via an ISDR non-dependent way (68-70). In addition to NS5A, also E2 and the IRES in the 5'UTR has been described to inhibit PKR and the downstream antiviral signaling (71, 72). Additionally, HCV has also developed strategies to render the host cell more tolerant against TNFα mediated apoptosis by the NS3/4A and NS5A proteins interfering with the TNFα signaling (73-75). Furthermore, the core protein of HCV is also involved in affecting the innate immune system by interfering with the STAT pathway and thereby inhibiting interferon stimulating genes (ISG) (76).

2.7.1.2 Natural killer (NK) cells

The liver consists of an unusual high frequency of NK cells with 30-50 % of the lymphocytes being NK cells compared to 5-20 % in the peripheral blood, which indicates the importance of this cell type in the liver (77). Natural killer cells play an important role in the rapid response to pathogens and tumors. The NK cells have the ability to rapidly kill target cells without antigen recognition or prior activation. Instead the NK cell recognizes abnormal levels of major histocompatibility complex (MHC) class I and/or stress induced molecules on the target cell (78, 79). The NK cells are present in two subgroups, a more mature form and a less mature form. The more mature form of NK cells has high cytotoxic effect with the ability of activating cell death receptors that mostly involve the tumor necrosis factor (TNF) superfamily such as Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL), TNFα and release of perforin and granzyme. The less mature variant of NK cells has the capacity of secreting granulocyte-macrophage colony-stimulating factor, IFNγ, TNFα, interleukin (IL)-5, IL-10, IL-13 and tumor growth factor (TGF)-β (80). During chronic HCV infection the NK cells have a dysfunctional phenotype and the overall number of NK cells are also decreased (81, 82). Beside that dysfunctional NK cells may not be able to directly kill HCV infected hepatocytes, their dysfunction also affects the cytokine production, which may also affect other cells ability to eradicate HCV. The Th2 type of response with decreased IFNγ production and increased production of IL-10 and TGF-β also influence and diminish the function of dendritic cells and T cells (83-85).

2.7.1.3 Natural killer T (NKT) cells

Natural killer T cells are a heterogeneous type of T lymphocyte that co-express the T cell receptor (TCR) and NK cell markers. These cells have both effector and immune regulatory function (86). The NKT cells have the ability to recognize both self and foreign lipids and glycolipids via the MHC class 1b molecule (CD1). Activation of NKT cells stimulates expression of FasL, secretion of cytokines like IL-4, IFNγ, TNFα and activation of other cells like dendritic cells (DC) and NK cells. This is suggesting that NKT cells may be involved in both eradication of virally infected cells and immune-mediated liver damage (87). Natural killer T cells have been shown to be reduced in numbers during chronic HCV infection and following sustained responses after IFNα/ribavirin treatment the number of NKT cells are increased (81, 88, 89). This indicates that the NKT cells are involved in the clearance

of HCV but on the other hand there has also been reports correlating the degree of fibrosis and liver damage with the frequency of activated NKT cells.

2.7.1.4 Dendritic cells (DC)

Dendritic cells are the connection between the innate and adaptive immune response and also the major antigen-presenting cell in the body. Dendritic cells are recognizing viral infection via pathogen-associated molecular patterns (PAMPs) after uptake of the pathogen and then migrate into the lymph nodes and present the antigen to specific T cells. Two main subgroups of DC exist, myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). Myeloid DCs are mainly associated with expression of TLR-3 and 8, antigen processing and presentation as well as secretion of IL-10 and IL-12. Plasmacytoid DCs are instead expressing TLR-7 and 9 as well as IFNα production. In patients with chronic HCV the mature function seems to be impaired as well as the frequency of mDC and pDC are reduced (90, 91). Myeloid DC in chronically infected patients are also impaired in the capacity of stimulating $CD4^+$ T cells and in the secretion of IL-12. The mDCs have increased expression of the exhaustion molecule programmed death (PD)-1 and the ability to produce IL-10 is increased as well as priming of IL-10-producing T cells (90, 92). These mDCs also seems to have the ability to impair the priming of HCV-specific T cells by inducing proliferation of regulatory T cells (Tregs) (93). The pDC in chronically infected HCV patients are also impaired regarding the function of producing IFNα (90, 94)

2.7.1.5 Kupffer cells (KC)

The liver is harboring a large group of cells important for phagocytosis and antigen presentation. This population is a type of macrophages only present in the liver and termed Kupffer cells (KC). The KCs are expressing high levels of CD80, CD40 and MHC class II molecule indicating the phenotype of a professional antigen-presenting cell and are upon phagocytosis of pathogens activated by TLRs as in hepatocytes and results in production of type I and III IFN as well as production of IL-1, IL-6 and TNFα (95). During chronic HCV infection the Kupffer cells express increased levels of galectin-9, which is the ligand for the T cell immunoglobulin domain and mucin domain protein (TIM)-3. The galectin-9 is of importance due to its effect on the expansion of Tregs, reductions of $CD4^+$ T cells and apoptosis of $CD8^+$ T cells and thereby the suppression of the HCV-specific T cell response (96). It has been shown that macrophages during chronic HCV infection are linked to impairment of IL-12 production, which is a key cytokine in the innate and adaptive immune response regarding differentiation of T cells and secretion of IFNγ and TNFα (96).

2.7.2 Adaptive immune response

The adaptive immune response is consisting of B cells that produce antibodies (e.g. plasma cells) and T cells. T cells are divided into two main groups, $CD4^+$ T cells (T helper cells, Th) and $CD8^+$ T cells (T effector cells, CTL). The adaptive immune system is activated during HCV infection by the infected hepatocytes or by specific antigen presenting cells (APC) like dendritic cells (DC), macrophages and B cells.

2.7.2.1 Activation of T cells

Endogenous pathway: In infected hepatocytes the viral proteins are degraded and processed in the cytosol as for all endogenous proteins. The proteasome processes the protein to peptides (8-10 amino acids) that are transported via the transporters associated with antigen processing (TAP) into the ER

where synthesized MHC class I are located and can recognize and bind the peptide. The peptide-MHC class I complex are then transported via the golgi apparatus to the cell surface were the peptide is presented to $CD8^+$ T cells. The $CD8^+$ T cells binds the MHC class I with its T cell receptor (TCR) and the CD8 molecule as co-receptor, which results in a signaling cascade activating the effector function (**Figure 6a**). (97)

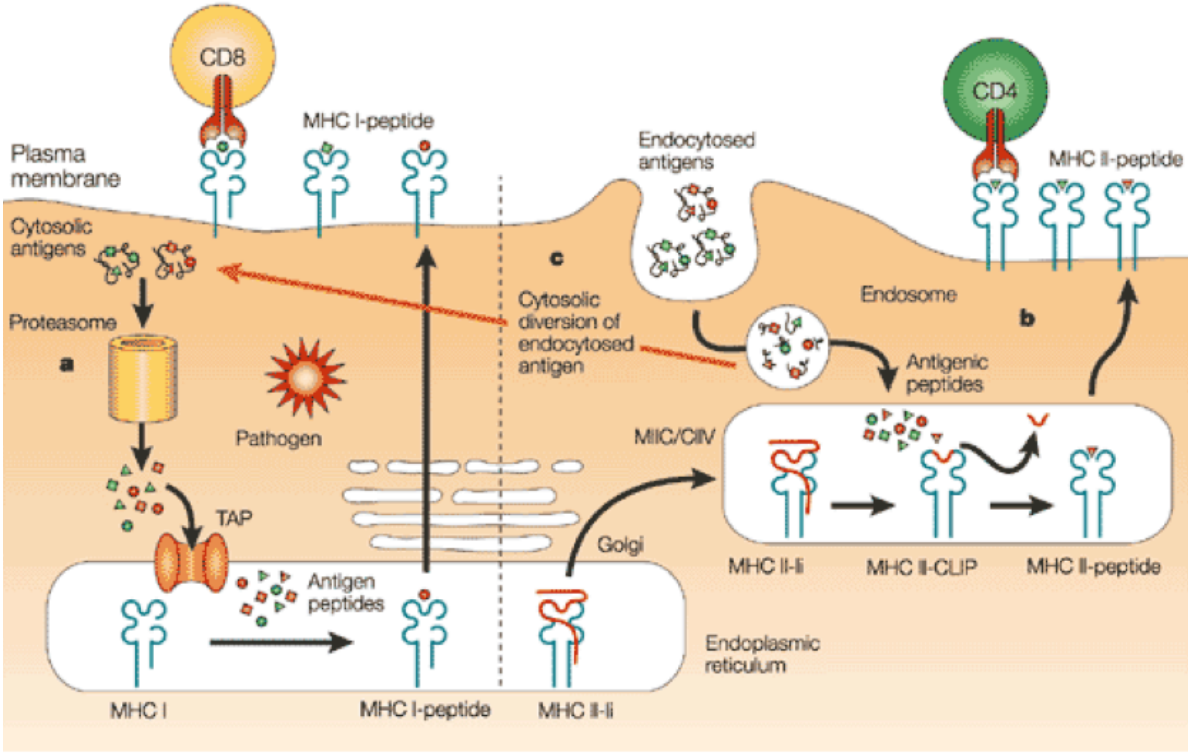

Figure 6. Illustration of antigen-processing and presentation via endogenous pathway (a), exogenous pathway (b) and by cross-presentation (c). Adapted by permission from Macmillan Publishers Ltd: [Nat Rev Immunol.] (98), copyright (2001).

Exogenous pathway: The activation can also occur via APCs (exogenous pathway) where dendritic cells, macrophages and B cells are engulfing cell or cell debris from apoptotic, necrotic or infected cells. The antigen that enters the cell from the exogenous pathway are processed into 13-18 amino acid long peptides in endosomes which are then fused with exocytic vesicles from the golgi apparatus encapsulating MHC class II molecules. Here MHC class II recognize and binds peptide, which are then transported to the cell surface where MHC class II can present the peptide for $CD4^+$ T cells. The $CD4^+$ T cells are recognizing the MHC class II via the TCR receptor and the CD4 molecule as co-receptor (**Figure 6b**). (97)

Cross-presentation or cross-priming: Exogenous antigen can also be presented on the MHC class I to $CD8^+$ T cells by cross-presentation. This occurs in DCs, which are professional antigen presenting cells and have the capacity of engulfing exogenous antigens and instead of having them processed by the exogenous pathway the antigens are released from the vesicle to the cytosol, and thereby entering the endogenous pathway and are presented on the MHC class I (**Figure 6c).** (97)

2.7.2.2 T cells

The T helper cells (Th) are activated by the exogenous pathway via the interaction between MHC class II and $CD4^+$ T cells. The activated $CD4^+$ T cells are then differentiating to either Th1 or Th2 cells characterized by cytokine secretion and function. The Th1 cells induce cellular immune responses by secretion of IL-2, IL-12, IFNγ and TNFα. The activated effector cells in a Th1 type of immune response are $CD8^+$ T cells or cytotoxic T lymphocytes (CTLs) and NK cells with the effector function of killing target cells using granzyme, perforin and/or the death pathway (Fas-FasL). The effector function of $CD8^+$ T cells is not restricted to the cytolytic functions mentioned above but also non-cytolytic effector functions mediated by secretion of cytokines as IFNγ and TNFα. The Th2 response is characterized by activation of the humoral immune response, represented by the B cells expressing/secreting antibodies upon secretion of IL-4, IL-5, IL-6 and IL-13. $CD8^+$ T cells recognizes antigen either by presentation of the MHC class I via the endogenous pathway or by cross-presentation and thereafter differentiate into effector cells (CTLs). (97)

Even though the HCV infection results in high levels of HCV replication the HCV-specific T cells first appears 4-8 weeks after the primary infection. Why the response is delayed is not completely understood but may be associated with the tolerant environment in the liver (99). Another explanation is the capability of HCV to avoid the innate immune system (discussed later). Resolution of acute and chronic HCV infections are associated with robust memory $CD4^+$ and $CD8^+$ T cells that have broad specificity to the HCV protein; core, NS3, NS4 and NS5. In addition, these individuals have increased plasma levels of IL-21 (100-104). It has also been shown that control of HCV infection is associated with a polyfunctional T cell response producing IFNγ and IL-2 (105). In patients who have spontaneously cleared an acute HCV infection, the $CD4^+$ and $CD8^+$ memory T cells could be detected in peripheral blood for up to 20 years in humans and 7 years in chimpanzees (106, 107). The importance of both $CD4^+$ and $CD8^+$ T cells has also been confirmed by depletion studies. Antibody-mediated depletion showed that in the absence of $CD4^+$ T cells, the $CD8^+$ T cells could control the viral replication initially, however low levels of viremia could still be detected and the functional $CD8^+$ T cells vanished (108). If instead the $CD8^+$ T cells were depleted the control of the viremia was impaired (106).

In chronic HCV infection the HCV-specific T cells are impaired in regards to several aspects. The $CD4^+$ T cells response that is critical for a sustained HCV-specific $CD8^+$ T cell response is often weak and production of IL-2 is reduced (109, 110). The cytotoxic effector function and the $CD8^+$ T cell proliferation are impaired as well as the production of IFNγ (111, 112). It has been shown that HCV-specific $CD8^+$ T cell responses during chronic infections are weak or undetectable in peripheral blood, whereas detectable levels are found in the liver characterized by increased expression levels of molecules associated with exhaustion (high PD-1, low CD127) (113, 114). Molecules TIM-3, PD-1 and cytotoxic T cell antigen 4 (CTLA-4) are associated with down-regulation of the cellular immune response. They are all highly expressed on intrahepatic HCV-specific $CD8^+$ T cells during chronic HCV infection but absent on $CD8^+$ T cells specific for cytomegalovirus or on $CD8^+$ T cells from patients who have resolved the HCV infection after antiviral treatment (115). Interestingly, the HCV-specific production of IFNγ, IL-2 and TNFα as well as the expansion of $CD4^+$ T cells could be restored by blocking of PD-L1/2, IL-10 and TGFβ (116). Furthermore, the functionality of T cells differs depending of the type of antiviral treatment. It has been shown that patients treated with PEG-IFNα/RBV still have dysfunctional and exhausted HCV-specific $CD8^+$ T cells (117). In contrary,

suppression of HCV replication by IFN-free treatments restores the HCV-specific T cell response during and after cure (118).

Regulatory T cells (Tregs) has several important mechanisms to down-regulate the immune response such as expression of IL-10, TGFβ, inhibitory molecules (PD-L1, galectin-9 and CTLA-4). The levels of $CD4^+/CD25^+$ T cells has been demonstrated to increase during chronic HCV infection and decreased to the same levels as healthy controls after cure (119, 120). Regulatory T cells have been described to alter the adaptive immune system during chronic HCV infection and are therefore believed to play an important role to maintain the infection.

2.7.3 Immune evasion

Beside the strategies mentioned above HCV may also escape the immune system by immune evasion due to the high mutation rate in the HCV genome. The NS5B protein or the RNA-dependent RNA polymerase lacks proofreading activity and this in combination with the high rate of viral replication causes introduction of mutations in the viral genome. There are several ways of how the introduced mutations can cause immune escape. The introduced mutations may lead to differences in the processed peptides after proteasomal degradation, which may result in that T cells recognize different peptides/epitopes (121). Mutations in epitopes may also result in loss of MHC peptide-binding or that the TCR do not recognize the epitopes. This results in that epitopes cannot be presented by the infected cell nor targeted by the T cells (122, 123). Mutations are an important mechanism for HCV to escape the host immune system, which has been shown with correlation between mutated $CD8^+$ epitopes and persistent infection as well as correlation between wild-type (non-mutated) $CD8^+$ T cell epitopes and clearance of the infection (124-126). At the same time as the mutations can help the virus escape from the immune system it can also affect the virus ability to replicate and produce new infectious viral particles (viral fitness). If a mutation affects the viral fitness too much that variant of the virus will vanish due to other virus variants with higher viral fitness. Some escape mutations is therefore less likely to appear in nature (127).

2.8 MODEL SYSTEMS FOR HCV

2.8.1 *In vitro* models

In the past, *in vitro* research has been limited due to lack of cell culture systems susceptible for HCV infection, replication and virus production. Primary hepatocytes from humans and chimpanzees have been utilized but only with limited success. The primary cells only allowed for low level of replication, a short life span (few passages) and contamination problems (128). Despite the best of efforts, for many years researchers were unable of using robust and reliable cell culture systems. In addition, numerous immortalized cell lines have been studied over the years without sufficient results. The current available cell culture systems are described below.

2.8.1.1 The sub-genomic replicon model

Ten years after the discovery of HCV the first replicon model was established as a sub-genomic replicon harboring the non-structural proteins essential for replication (129). The year after, a replicon system containing the complete HCV genome was established (130). The replicon system allowed studies of host-viral signaling pathways and to study how mutations affect HCV replication. Adaptive mutations promoting HCV replication were found in the NS3, NS5A and NS5B proteins, which

strongly increased the levels of RNA replication (130-132). The sub-genomic replicon system has been invaluable for development and screening of HCV direct-acting antiviral drugs. However, the replicon system does not allow studies of viral entry and assembly.

2.8.1.2 The HCVpp model

The HCV pseudoparticle (HCVpp) model was developed to study entry and fusion of HCV into cells. The HCVpp is based on the retroviral proteins Gag and Pol, involved in the packaging of RNA and budding of virus particles, using the HCV glycoproteins as membrane of the produced virus particles. Human embryonic kidney cells (293T) were transfected with three vectors. The first vector includes retroviral Gag and Pol. The second vector codes for a reporter gene (luciferase or GFP). The third vector encodes for the HCV proteins E1 and E2. Pseudoparticles produced in 293T cells could then be used for infection of Huh-7 cells and this model became soon essential for studying neutralization and fusion mechanisms *in vitro* (22). This model has been a valuable tool in identification of the HCV co-receptors Claudin-1 and Occludin. It has been shown, by using this model, that monoclonal antibodies against E1 and E2 protein as well as sera from infected patients could neutralize entry of the pseudoparticles (22, 53, 133). The HCVpp model has been vital for understanding binding, attachment and entry, and this model is still important for studying neutralization antibodies and viral entry.

2.8.1.3 The HCVcc model

In 2005 there was a major breakthrough in the research field of HCV. A HCV strain isolated from a patient with fulminant hepatitis in Japan was shown to replicate in cell culture and could also produce infectious virus particles in the supernatant (HCVcc) (134-136). The Japanese fulminant hepatitis 1 or JFH-1 was of genotype 2a, a unique clone with capacity of infecting hepatoma cells, chimpanzees and mice transplanted with human hepatocytes. Unlike HCVpp, the HCVcc could infect hepatoma cells resulting in production of infectious virus particles with the ability to infect naïve hepatoma cells. Since the discovery of JFH-1 several HCVcc constructs have been generated for different HCV genotypes in cell culture (137). The establishment of the HCVcc system now allows researchers to study all steps in the HCV life cycle as well as neutralization and the effects of antiviral drugs.

2.8.2 *In vivo* models

2.8.2.1 Chimpanzee

Chimpanzees are together with humans and the Tupaia (Three shrew) the only known species that are susceptible to HCV infection. For a long time the chimpanzee was the only model to study HCV and the model has been valuable in the discovery of the virus and for research about viral replication, transmission, and immune responses. Although the chimpanzee model has been of great value, it is also associated with high cost, limitation in the quantity of animals that can be used, and ethical problems. Other barriers are that humans and chimpanzees do not share HLA class I, and there are also observed differences in MHC class II (138). In addition, the disease progression differs between humans and chimpanzees with a lower frequency of infected chimpanzees that progress to chronicity. Also chimpanzees do not show signs of liver disease or tissue damage, which makes it problematic to study cirrhosis and hepatocellular carcinoma in this model (139, 140).

2.8.2.2 *HCV transgenic mice*

Several mouse strains transgenic for HCV proteins have been generated over the years for studying *in vivo* effects of the different HCV proteins. All HCV transgenic mice strains have the genetic material for HCV integrated into the genome and depending on the promoter expressed in all tissues or in selected tissues/organs. There have been some contradictive results regarding HCV-associated pathology in the transgenic mice where for example the core protein has been shown to induce steatosis and hepatocellular carcinoma in one study and in another study they found no pathological effects of transgene expression (141-143). The discrepancies may be explained by the use of different transgenic mouse lineages with different chromosomal integration of the transgene, expression levels and the genetic background of the mouse. The effects of the transgene-expression on the innate and adaptive immunity have also been studied. Two independent studies showed that transgenic expression of NS5A and NS3/4A affects the TNF-mediated hepatic apoptosis (73, 144). HCV transgenic mice have also been valuable in studies of vaccine development where the main purpose of the inserted transgene has been to mimic a chronic HCV infection with immune tolerance to the HCV proteins. However, there are some aspects to remember and consider when using transgenic mice. The transgene-expression and antigen processing may not be the same as during the natural infection. The protein-expression levels can be lower, undetectable or higher. Immune tolerance as a result of the transgene-expression can be a problem for studying immune responses and thus it is of importance to choose how the protein/s are expressed. Immune tolerance can possibly be avoided with delayed expression e.g. onset after birth (mouse major urinary protein (MUP) promoter) or if using an induced expression (Cre-Lox). Limitations with transgenic mouse models are that they take long time to generate and breed. The data generated from transgenic mice may be difficult to interpret but if the results are evaluated with caution the transgenic mice can generate important information.

2.8.2.3 *Transiently transgenic mice*

Transiently transgenic mice can be generated in any mouse strain by an intravenous injection in the tail vein. The technique was described for the first time in 1999 and was referred to as hydrodynamic injection (145). A hydrodynamic injection is based on a rapid (5-20 seconds) injection of a large volume (1,6-2,0 mL) of plasmid DNA solution in the tail vein. This injection leads to a high pressure in the liver and causes enlargement of the sinusoidal fenestrae. This causes an extravasation in the liver and by that increased permeability of the hepatocytes, 10 to 40 % of the liver cells are transfected (146). This model is valuable for studies of transient expression of proteins in the liver. Results from the transiently transgenic mice may also be compared with data obtained in stably transgenic mice. In the transiently transgenic model, the eradicated hepatocytes are replaced by a new non-transgenic hepatocyte. This means that one can use this model for studying clearance and the functionality of immune responses primed by immunization. Hence, this model allows evaluation of vaccine efficiency. It has been shown that by using this model NS5A-specific cytotoxic T cells primed in the periphery could enter the liver and eradicate NS5A-expressing hepatocytes (**paper IV**). The advantage of the model is that any mouse strain can be made transiently transgenic for an antigen with peak protein-expression levels at 48-72 hrs after injection. The main drawback is that the hydrodynamic injection causes transient liver damage evidenced by elevated ALT levels in serum.

2.8.2.4 *Genetically modified animals*

Wild-type mice cannot be infected by HCV but the mouse genetics can be manipulated, by knocking down or complementing the mouse with human exogenous factors vital for the viral life cycle. As

described earlier, the human receptors CD81 and OCLN are essential for entry of HCV into mouse hepatocytes, which was recently tested in an genetically modified mouse (50). Mice with impaired innate immunity (STAT1-/-, IRF1-/-, IRF7-/-) and transgenic for the human entry factors (CD81, SR-B1, CLDN1 and OCLN) were permissive for HCV infection, and allowed replication as well as virus production for two months (147). This is the first partially immuno-competent mouse model that recapitulate the entire virus life cycle. This model may possibly be used for evaluation of antiviral drugs.

2.8.2.5 Mouse models with humanized liver

Humanization of the mouse liver has been one procedure to recapitulate the HCV life cycle in a mouse. Several mouse strains has been established e.g. uPA-SCID, FRG, MUP-uPA and HSV-TK where liver injury is induced by different techniques and thereafter repopulated with human primary hepatocytes (148-152). The advantage with this mouse models is that they contain human hepatocytes and are permissive for HCV infection (153). The main weakness is that the mice are immune deficient in order to avoid the rejection of the engrafted human hepatocytes. Since these models lack a functional immune system they are not suitable for vaccine studies. However, these models are still valuable for studies of the viral life cycle and evaluation of antiviral drugs.

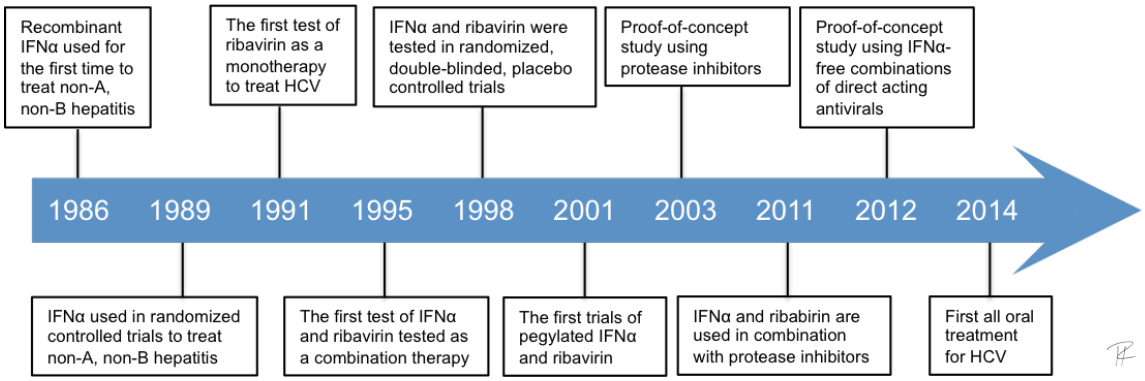

Figure 7. Schematic illustration over the treatment of hepatitis C virus infection.

2.9 TREATMENT

2.9.1 Antiviral therapy

The first treatment of HCV started already 1986 before HCV was identified and still named non-A non-B hepatitis. At this time the treatment for HBV was recombinant IFNα and it exerted some anti-HBV effects. This treatment was tested for HCV as well and resulted in a cure rate of approximately 6% (154-156). During the 1990s several studies regarding the dose and length of IFNα treatment resulted in adjustment of the treatment length to 48 weeks instead of 24 weeks and the cure rate increased to 16% (157). Monotherapy using ribavirin (RBV) was tested in the beginning of the 1990s and resulted in lower alanine aminotransferase (ALT) levels but had no effects on the HCV RNA levels (157-159). In the second half of the 1990s the first combination therapy and randomized trials were carried out for HCV and published. This treatment consisted of IFNα and RBV, which increased

the sustained virological response (SVR) after 24 and 48 weeks of treatment to approximately 34% and 42% respectively (160-164). Further development of the treatment, including increasing the half-life of IFNα by pegylation (PEG-IFNα), resulted in around 39% of the patients achieving SVR after 48 weeks of treatment and when used in combination with RBV SVRs of 54% to 56% were achieved (165-167). Additional optimizing since the year 2000 with PEG-IFNα adjusted to the patients weight on weekly basis and a fixed dose of RBV daily. Clinical trials based on PEG-IFNα and RBV reveled that; genotype 2 and 3 was associated with higher cure rate (76-82%) compared to genotype 1 (42-46%), HCV RNA levels influenced the outcome of treatment with cure rates of 78% if viral load was $<2x10^6$ IU/mL and 42% if viral load was $>2x10^6$ IU/mL, and that genotype 2 and 3 could be treated during 24 weeks instead of 48 (168-170). At this time one knew that host factors were correlated to successful treatment response, female sex, young age, low fibrosis level and non-African American heritage were all in favor of clearing the infection (171-174). It took until 2003 to identify and test the first direct-acting antivirals (DAAs). The first ones were NS3 protease inhibitors (175). More than 20 years after the molecular identification of HCV, DAAs became used (one protease inhibitor, Boceprevir or Telaprevir), in combination with PEG-IFNα and RBV to treat HCV infected patients (176, 177). Notably, the protease inhibitors were specific for genotype 1 and the cure rate reached around 75% (176, 177). Due to the severe side effects caused by INFα and RBV the goal has been to develop INFα-free treatment and one step was taken in 2012 with the first proof-of-concept study of an IFNα-free combination treatment using the NS3 protease inhibitor Asunaprevir and the NS5A inhibitor Daclatasvir (178). The development of new DAAs has developed rapidly with several DAAs on the market and several others are in clinical trials (**Figures 7-8**) (179-181).

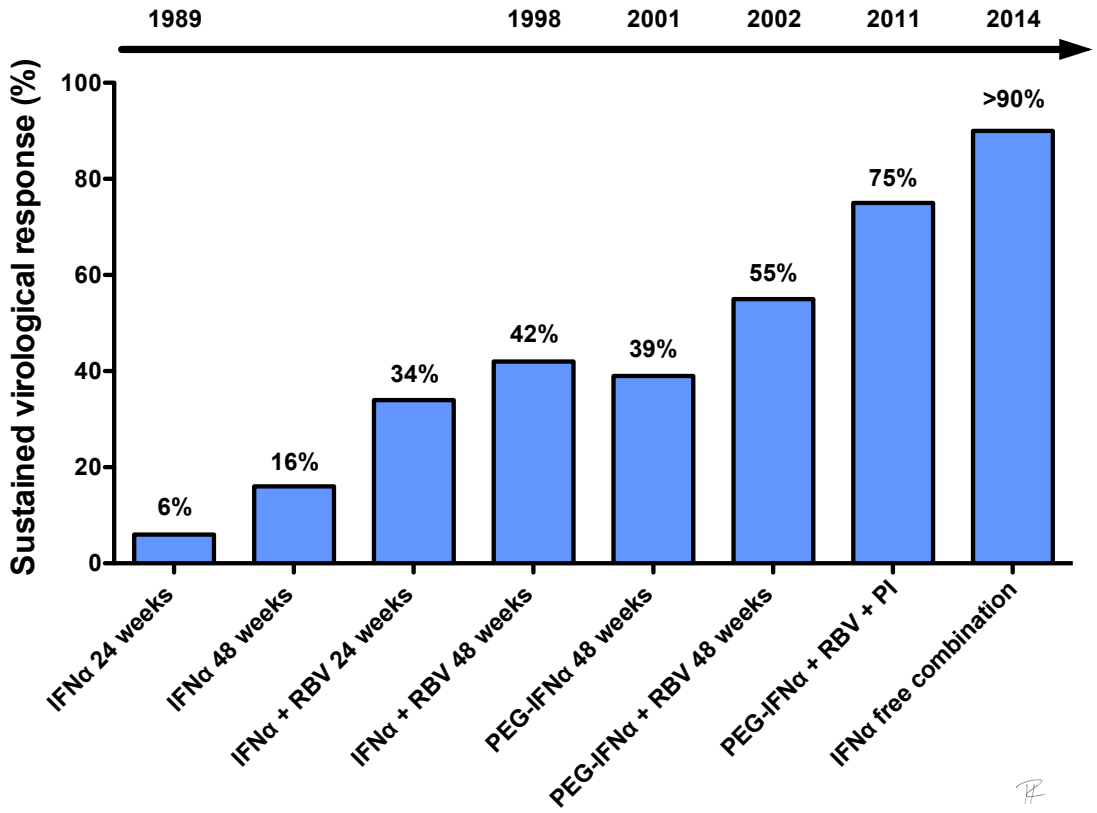

Figure 8. Treatment success rate over the years.

The goal of an IFNα and RBV free treatment with 100% SVR are soon reached in an all oral IFNα and RBV free regimen using Ledipasvir (NS5A inhibitor) and Sofosbuvir (NS5B inhibitor) and a triple therapy with Paritavir (NS3 protease inhibitor), Ombitavir (NS5A inhibitor) and Dasabuvir (NS5B inhibitor) with SVR rates >90% (181, 182). The newly introduced DAAs cure most of the treated patients, however the new treatment do not protect against re-infection. Another limitation with the new DAA treatment is the high cost, which today limits the use to patients with severe liver disease. Another limitation with the DAAs is the risk of developing drug-resistant variants. Also, it is debated whether patient groups like immunosuppressed, liver-transplanted, children and pregnant women will be eligible for treatment using the new DAAs.

One strategy to combat HCV would be to develop a prophylactic vaccine, which not only protect humans against HCV infection but also is economically beneficial for the society. An alternative treatment strategy for patients with HCV would be to combine DAAs with an immune stimulatory treatment that could also prevent patients from re-infection. Important to remember is that only 10% of all HCV infected patients receive any treated today (183). Hence, there are unmet needs for new treatment strategies that are associated with lower costs to make it possible to treat the remaining 90% (mostly found in low-income countries). Thus, research for alternative therapies should continue.

2.9.1.1 Interferon-α

Interferon-α (IFNα) is an important molecule in the innate immune system upon infection of intracellular pathogens. The signaling of IFNα is both autocrine and paracrine to induce an antiviral state of the infected and neighboring cells after binding to its receptor. The signaling cascade starting from the interferon receptor via the JAK-STAT pathway and results in induction of interferon stimulatory genes (ISGs). The IFNα does not only induce an antiviral state with promoted RNA degradation and inhibited protein synthesis but also activation of immune cells. The pegylated IFNα that is used today has a polyethylene glucol molecule conjugated to IFNα. This modification increases the half-life of IFNα and thereby reduces the number of injections needed (165, 184). The IFNα is associated with numerous side effects including severe fever, apathy, irritably, diarrhea and severe depression.

2.9.1.2 Ribavirin

Ribavirin (RBV) is a guanosine analoge with broad antiviral activity against several viral pathogens. It has been described that RBV has immuno-modulatory effects affecting the T helper (Th) 1/Th2 ratio by shifting it to a Th1 response (185-187). The antiviral genes induced by IFN have also been reported to be up regulated by RBV (188-191). The side effects induced by RBV are severe skin rash and hemolytic anemia.

2.9.1.3 Direct-acting antivirals (DAAs)

Protease inhibitors are targeting the NS3 serine protease of HCV. The first DAAs that were approved as treatment were Boceprevir and Telaprevir, which are both protease inhibitors (176, 177). The development of new NS3 protease inhibitors has generated improved pharmacokinetics and tolerability compared to Boceprevir and Telaprevir. Simeprevir was the third approved protease inhibitor for use in combination with IFNα and RBV (192-194). Simeprevir has also been tested together with Sofosbuvir in an IFNα free setting (195). Paritavir (ABT-450) is also an approved protease inhibitor for use in combination therapy with Ombitavir (ABT-267) and Dasabuvir (ABT-

333) as an all-oral IFNα free treatment regimen (196, 197). Several other protease inhibitors are on the way to reach the market such as Grazoprevir (MK-5172), Asunaprevir (BMS-650032) and Faldaprevir (198-204)

Nucleoside/nucleotide analogues are inhibitors that inhibit the NS5B protein, which is the RNA-dependent RNA polymerase. The incorporation of this analogue results in either termination or introduction of mutations in the synthesized viral RNA genome. Sofosbuvir, an approved drug for treatment of HCV belongs to this group of DAAs and has been shown to achieve high cure rate in an IFNα free treatment alone and in combination with Ledipasvir and Simeprevir (195, 205-208). Sofosbuvir also seems to have pan-genotypic activity (209).

Non-nucleoside analogues are instead of being incorporated in the RNA genome targeting the active domains of the RNA-dependent RNA polymerase and by that inhibiting its function. Included in this group is BMS-791325 and Dasabuvir (ABT-333). BMS-791325 has been reported to work in a combination study with Daclatasvir and Asunaprevir (201). Dasabuvir (ABT-333) has been approved as an all-oral combination with Paritavir (ABT-450) and Ombitavir (ABT-267) (196, 197).

NS5A inhibitors bind to NS5A and interfere with its function during replication and assembly. The first DAAs targeting NS5A was Daclatasvir (BMS-790052), which has been tested in combination therapies with PEG-IFNα and RBV and as an all-oral combination with Asunaprevir and BMS-791325 (200, 201). Ledipasvir (GS-5885) has been tested in combination with Sofosbuvir in an all-oral IFNα free treatment with promising results (205, 206). Ombitasvir (ABT-267) also belonging to this group has been approved in combination with Paritavir (ABT-450) and Dasabuvir (ABT-333) (196, 197). Other examples of NS5A inhibitors under development are Elabasvir (MK-8742) and GS-5816 (204, 210, 211).

Host target antigens are targeting the host factors required during the HCV life cycle. They have a high barrier to resistance and are pan-genotypic, which is advantageous. Alisporivir (Debio-025) is one host target antigen that targets Cyclophilin A and by that inhibiting the association with NS5A (212, 213). Other host proteins that are interesting targets for antiviral drugs are mir122 (a micro RNA important for HCV replication) and SR-B1 (one of the HCV entry receptors) (214, 215)

2.9.2 Vaccines against HCV

There are several different kinds of vaccines today. Inactivated pathogens are used to induce protective antibody responses, which also are raised using proteins and protein subunits vaccines. Live attenuated vaccines are the most effective vaccine, which activate the immune system in a natural way. Genetic vaccines encoding for proteins or peptide administered as naked DNA or by using viral vector try to mimic a natural infection and by that induce an immune response (216). Vaccines can be divided in two different groups, prophylactic and therapeutic vaccines. The aim of prophylactic vaccines is to activate a strong, specific and effective immune response mainly focused on B cells (antibodies) that can neutralize the virus. Preferentially the immune activation should be remembered (immunological memory) meaning that Th cells are also of importance. The immunological memory is of vital importance for a vaccine to be able to rapidly recall the immune response when infected by a pathogen. A therapeutic vaccine on the other hand is aiming to activate an already existing sub-optimal or exhausted immune response. The focus for a therapeutic vaccine is to activate a robust, strong, specific and effective immune response, mainly cytotoxic T cells to eradicate infected cells, preferably with an immunological memory to prevent re-infection. There are several both prophylactic

and therapeutic vaccines under development for HCV. Discussed below is the current status of the development of HCV vaccines.

Chiron/Novartis (Emeryville, CA/ Basel, Schweiz) are developing a protein-based prophylactic vaccine including two structural proteins e.g. envelope protein 1 and 2 originating from genotype 1a. The vaccine is delivered in combination with the adjuvant MF59, which is an organic 30-carbon compound that stimulates $CD4^+$ T cells. The results from a clinical phase I trial (13 healthy volunteers) show induction of cross-neutralizing antibodies that could be detected in 12 out of 13 individuals. Interestingly, in 2 out of 13 individuals cross-neutralizing antibodies against all 7 major genotypes could be detected (217).

Okarios (Rome, Italy) are developing a prophylactic/therapeutic vaccine based on the non-structural proteins NS3, NS4, NS5A and NS5B of genotype 1b. The vaccine is delivered using viral vectors in a heterologous prime-boost regimen by priming with a replicative deficient simian adenovioral vector (ChAd3) and boost with a Modified Vaccinia Ankara vector (MVA). The vaccine activates high levels of HCV-specific $CD4^+$ and $CD8^+$ T cells targeting multiple epitopes in a phase I clinical trial (15 healthy volunteers). Boosting with MVA resulted in improved proliferation, polyfunctionality and memory responses (218).

Transgene (Strasbourg, France) are developing a vaccine based on MVA including the NS3, NS4 and NS5B proteins of genotype 1b. The vaccine named TG4040 has been tested in a phase II clinical trial where HCV infected patients were given PEG-IFNα and RBV (n=31), PEG-IFNα, RBV and TG4040 (n=63) or start with TG4040 and then PEG-IFNα, RBV and TG4040 (n=59). Priming with TG4040 followed by the combination of PEG-IFNα, RBV and TG4040 resulted in the highest sustained virological response of 58,2%. Treatment with PEG-IFNα and RBV resulted in sustained virological response of 48,4%. In the group where TG4040, PEG-IFNα and RBV were administrated simultaneously sustained virological responses were achieved in 50,8% of the patients (219).

ChronTech Pharma AB (Huddinge, Sweden) is developing a vaccine named ChronVac-C that is based on NS3 and NS4A of genotype 1a. The vaccine is delivered by an intramuscular injection in combination with *in vivo* electroporation. The combination of DNA vaccination followed by PEG-IFNα and RBV treatment were tested in 12 patients and resulted in that 75% of the patients achieved a sustained virological response (220). This can be compared with 50 % sustained virological response in patients only treated with PEG-IFNα and RBV (165-167).

The vaccine developed by Transgene (TG4040) and ChronTech Pharma AB (ChronVac-C) has been tested in combination with two doses of ChronVac-C followed by three doses of TG4040 five to 12 weeks later in mice. The results of DNA-prime/MVA-boost showed significantly increased immune responses compared to administration of the two vaccines alone. High levels of polyfunctional T cells and induction of strong IL-2 and IFNγ levels as well as high percentage of $CD8^+$ T cells specific for HCV were detected. This was a proof-of-concept study proving that the combination of two clinically evaluated therapeutic vaccines were better than using the vaccines individually (221).

Inovio (Playmouth Meeting, PA) is developing a therapeutic vaccine based on a consensus sequence of genotype 1a and 1b. The vaccine is a DNA vaccine composed of the NS3/4A, NS4B, NS5A and NS5B in four different constructs administrated via intramuscular immunization in combination with *in vivo* electroporation. The vaccine has been tested in Rhesus Macaques (n=4) resulting in broad

responses to multiple HCV epitopes and polyfunctional T cells (222). The INO8000/VGX-6150 is currently tested in a phase I clinical trial in Korea where the NS5B gene has been excluded and NS3/4A, NS4B and NS5A are combined in one vector (223).

Center for Genetic Engineering and Biotechnology (Havana, Cuba), is developing a vaccine named CIGB-230 based on the core protein and a plasmid expressing the envelope proteins E1 and E2 of genotype 1b have been tested in a phase I clinical trial (n=15). The results showed priming of HCV-specific T cells responses as well as secretion of IFNγ in 73% of the previously non-responding patients after initial immunization (24 weeks). At the end of the treatment 46,7% of the patients had cellular immune response to more than one HCV protein (224).

The above mentioned studies reveals the current status of the vaccines being developed for HCV. The vaccines developed by Chiron/Novartis and Okarios seems at the moment most promising. However, future studies will show if the vaccines live up to the expectations regarding immunogenicity and protection.

3 GENETIC VACCINES

There are different vaccine strategies that can be used to activate the immune system. Inactivated vaccines, subunit and protein based vaccines can be used and are able to induce strong B cell responses with production/secretion of antibodies with the ability of neutralizing the pathogen. The ability of inducing cellular immune response is crucial for eradication of intracellular pathogens but is often poor using these types of vaccines. Live-attenuated vaccines are based on weak variants of still functional viruses. The virus can infect and replicate in the host cell, which results in a natural activation of the immune system via pathogen-associated molecular patterns (PAMPs) recognized by pathogen recognition receptors (PRRs) (225). The immune activation following vaccination using a live-attenuated vaccine is potent with long-term protection, often life long e.g. measles, mumps and rubella vaccine (226). The use of live-attenuated viruses, as a strategy for a HCV vaccine is not possible due to safety concerns. The high mutation rate for HCV could result in that the weak strain is reverting back to a wild-type strain and establish a persistent infection. This type of strategy is therefore not suitable for pathogens with high mutation frequency. Genetic vaccines are also using the strategy of intracellular production of the vaccine antigen and are aiming at activating the immune response using the natural way as intracellular pathogens. One way to deliver genetic vaccines is by using viral vectors, which has been shown to induce effective immune responses in clinical trials e.g. Adenoviruses, Modified Vaccinia Ankara (MVA) (218, 219). The major challenge with viral vectors is the stability of the produced vaccine and problem with pre-existing immunity. Pre-excising immunity to the viral vector can limit the activation of immune response to the vaccine antigen. This can also be a problem if using the same vector for boosting as where used for the primary injection. The use of a heterologous prime-boost strategy may resolve this problem (218). Viral vectors have also shown effective boosting of the immune response after DNA immunization (221).

3.1 EUKARYOTIC EXPRESSION VECTORS

The DNA vaccine vector is basically a plasmid with bacterial backbone and a eukaryotic expression unit. The bacterial backbone is consisting of an origin of replication and a resistance gene that is needed to be able to produce the plasmid in bacterial cultures. The eukaryotic expression cassette

contains an eukaryotic promoter, Kozak sequence, the vaccine gene(s) and a poly adenylation signal. Regarding promoter there are different ones that can be used depending on the purpose. One often used promoter is the human cytomegalovirus (CMV) immediate-early promoter, which induces high constitutive expression in mammalian cells (227). This promoter is transcribed in most cells but with one drawback, it is down regulated by the cytokine IFNγ as a result of immune activation by e.g. CpG motifs after DNA vaccination (228, 229). Other alternatives are to use tissue-specific promoters. The use of tissue-specific promoters has both advantages and disadvantages. The expression of the vaccine-antigen is only located in the targeted tissue, which is an advantage. On the other hand the vaccine genes are commonly expressed in lower levels, which could be a disadvantage (230, 231). The Kozak sequence is important for the translation of the gene and the poly adenylation (polyA) signal is essential for the stability and thereby the expression of the vaccine gene (232, 233). A widely used polyA signal for DNA vaccines originate from the bovine growth hormone gene (BGA).

One important factor correlated to expression and immunogenicity regarding DNA vaccines is codon optimization. It is a difference in codon usage in different organisms and by adapting the codon usage of the vaccine gene to the most commonly used codons for amino acids in humans one can increase both the expression levels and immunogenicity of the vaccine gene (234-237).

3.2 DNA VACCINES

The era of naked plasmid DNA as vaccines started during the early 1990s. It began with the first injection of plasmid DNA generating protein-expression in mice the year of 1990 (238). Short after, humoral and cellular immune activation following injection of plasmid DNA was reported (239-242). In 1998 came the first report regarding DNA vaccine trials in humans with a vaccine against HIV-1 based on the Env and Gag proteins (243). The strategy has now been tested for several pathogens like influenza, HIV, HCV and cancer (244). Although 25 years of research has past there is still no DNA vaccine approved for humans. There are two approved vaccines against viral infections for use in animals, one for horses (Nest Nile virus) and one for salmon (infectious hematopoietic necrosis (245, 246). So why are there no approved DNA vaccine for humans? The main reason is that the vaccine does not active enough potent immune response in humans. The immune response could be strong and protective in small animal models but low when tested in humans. The reason is most probably the low uptake of DNA into the cells after injection. This results in low levels of antigen expressed in the cells. Beside this drawback one other concern has been discussed, which is the integration of the DNA vaccine into the host genome. So, why still put effort into the research of DNA vaccines? The answer is that the positive sides of DNA vaccines are many and the negative are fewer. To overcome above mentioned obstacles, the research has focus on optimizing the plasmid constructs, delivery techniques and adjuvants. Regarding genome-integration it has been shown to be a minor concern because the spontaneous mutation frequency in the human genome is much higher than the integration rate of vaccine DNA into the humans genome (247-250). Advantages of DNA vaccines are the low cost of production and easy storage of the vaccine. Developing a vaccine with low cost of production and an easy storage at room temperature or even at higher temperatures makes it possible to use such vaccines also in low-income countries. The fact that DNA vaccines are considered safe with few side effects (local irritation and inflammation at the site of injection) and with a preferred type of immune activation (both humoral and cellular) highlights the importance to continue the development of DNA vaccines for humans (244).

3.3 IMMUNE RESPONSE FOLLOWING DNA IMMUNIZATION

After injection of plasmid DNA it will be taken up by different cell types depending on the injection technique and site of injection (muscle, skin, mucosa and/or antigen presenting cells). Inside the cell the plasmid DNA is transported into the nucleus where the vaccine gene (in the eukaryotic expression vector) will be transcribed and translated. The expressed vaccine protein will be degraded in the cytosol, processed by the endogenous pathway and presented to $CD8^+$ T cells via the MHC class I molecule, which almost all cells in the body express. The vaccine protein can also be presented at MHC class II on antigen presenting cells after engulfing the protein. Phagocytosis of vaccine protein released from apoptotic, necrotic or vaccine protein expressing cells are degraded and processed by the exogenous pathway and presented to $CD4^+$ T cells via the MHC class II or via cross-presentation to $CD8^+$ T cells via the MHC class I molecule (**Figure 9**). (251, 252)

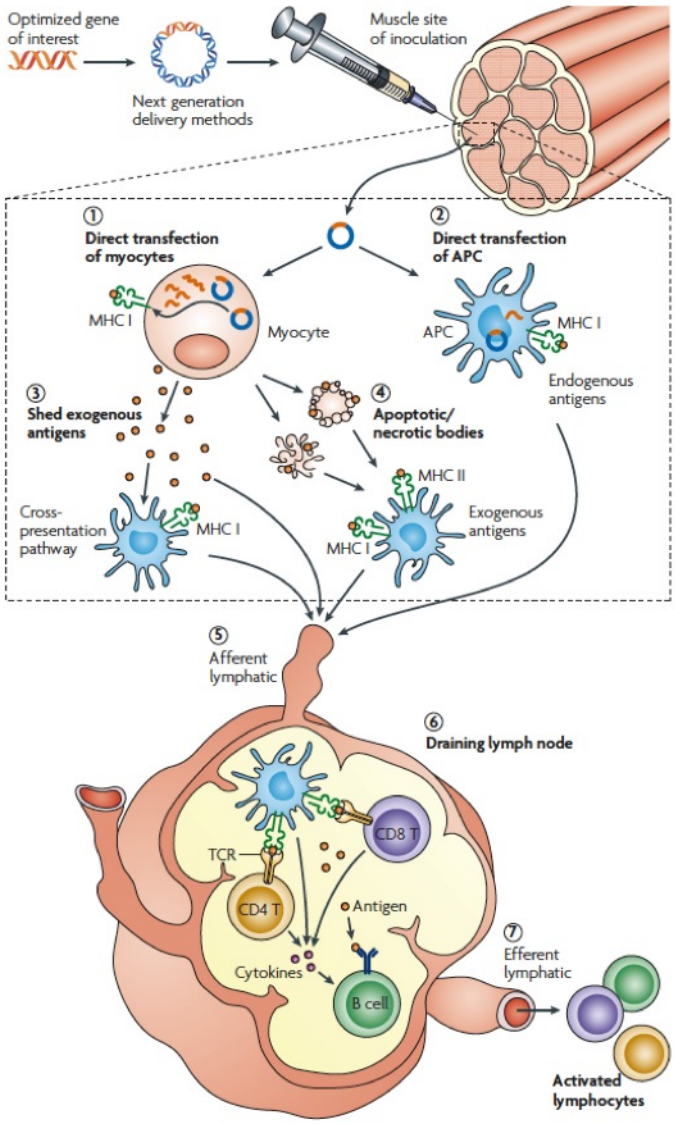

Figure 9. Priming of humoral and cellular immune responses by DNA immunization. Adapted by permission from Macmillan Publishers Ltd: [Nat Rev Genet.] (252), copyright (2008).

3.4 DELIVERY

Several different techniques have been developed and used to optimize the delivery of DNA vaccines. The choice of delivery technique is one way to target a certain type of tissue and thereby activate a preferred type of immune response. The aim of the delivery techniques is also to increase the uptake of DNA vaccine into the cell and thereby increase the expression levels of the vaccine antigen. The delivery technique may differ in efficiency depending on which species it is tested in. Also, different vaccine-antigens may require different delivery techniques to obtain an optimal immune activation. There are different types of delivery techniques e.g. intramuscular injection, intradermal injection, biojector, microinjection, gene gun and *in vivo* electroporation (253). Delivery using intramuscular injection, *in vivo* intracellular injection device (IVIN), *in vivo* electroporation and prime-boost regime will be discussed in more detail below.

3.4.1 Intramuscular injection (i.m.)

Intramuscular injection of plasmid DNA is a simple procedure often performed using a mixture of DNA in phosphate buffered saline (PBS) solution. After injection, the myocytes passively take up the plasmid DNA and express the protein. The protein will then be presented as peptides on the MHC class I molecule on the cell surface of myocytes to T cells. Alternatively, the vaccine-antigen is taken up by antigen presenting cells, which migrate to the lymph nodes and present it on the MHC class II or via cross presentation on MHC class I to T cells (97). In smaller animals such as mice it is well known that a large injection volume creates a hydrodynamic pressure in the muscle, which will increase the DNA uptake (254). In addition, myocytes inefficiently present antigens due to low levels of MHC class I expression and lack of co-stimulatory molecules. To increase the immunogenicity of DNA vaccines in larger animals and humans the administration technique needs to be improved.

3.4.2 *In vivo* intracellular injection device (IVIN)

We have tried to improve the intramuscular injection technique by developing the *in vivo* intracellular injection device or IVIN. This injection device consists of 4-5 needles with sealed needle tips. Instead the shafts of the needles have holes directing the injected vaccine-solution to a defined area. The DNA solution is injected using a fixed pressure and targets a defined area, which standardizes the technique. The high-pressure obtained using this injection device increases the uptake of the DNA resulting in enhanced expression of the vaccine-antigen (255) (Ahlén et al., manuscript in preparation, Levander et al, submitted manuscript).

3.4.3 *In vivo* electroporation (EP)

The use of electroporation to transfect DNA into cells were first described in the 1980s (256, 257). The use of i.m. injection in combination with *in vivo* electroporation can enhance the uptake of plasmid DNA in skin and muscle cells and increase the expression of the vaccine-antigen. It has been debated if the DNA passively enters the cells through the pores created by the electroporation or if it is also transported into the cells through the pores due to the electric field (electrophoretic effect). Studies have shown that electrotransfer of drugs and DNA works differently (258). The pores created by the electroporation mediates that drugs are passively diffusing through the membrane but DNA seams to be to large to passively diffuse through the pores. DNA injected after electroporation while the cells are still permeabilized does not results in transfection of DNA into the cells, which indicates that the electric field is important to transfer the DNA into the cells (259). DNA is highly negatively charged polyanions that mediates the DNA to move in an electric field. The permeabilization of the

cell membrane and the electrophoretic effect are both important for the uptake of DNA by electroporation. Pulse pattern for optimal uptake of DNA when using electroporation is one short high-voltage pulse that permeabilizes the cell membrane and one long low-voltage pulse for DNA transfer (e.g. one pulse 0.1 ms, 800 V/cm and one pulse for 400 ms, 80 V/cm) (260).

The parameters that can be changed in electroporation are the voltage, the number of pulses, the duration of the pulses and the time between the pulses. These parameters are essential for optimizing the delivery of DNA into cells by electroporation. The induction of cell-death and inflammation at the site of DNA immunization is important for a strong immune activation. The *in vivo* electroporation act as an adjuvant by enhancing the inflammatory response and infiltration of immune cells (261-263).

Studies have shown that the integration rate is higher when comparing i.m. injection plus *in vivo* electroporation with i.m. injection alone (264, 265). However, the cells that are at an increased risk are though the cells that have high-uptake of the DNA. These cells are expressing high-levels of the vaccine antigen and thus most often efficiently eradicated by the activated host T cell response (263).

Intramuscular injection combined with *in vivo* electroporation in skin and muscle has been shown to increase the uptake of plasmid DNA compared to regular i.m injection. Both humoral and cellular immune response has been shown to be effectively improved by the addition of *in vivo* electroporation in different animal models (e.g. mice, rabbits, rhesus macaques, pigs) (263, 266, 267). The use of *in vivo* electroporation when administering DNA vaccines allows the use of lower DNA doses because of more efficient uptake of the DNA. This is important to enable delivery of realistic DNA vaccine doses to humans. Additionally, the combination of the IVIN device and *in vivo* electroporation furthermore increases the uptake of plasmid DNA, which results in enhanced immune responses (Ahlén et al., manuscript in preparation).

3.4.4 Prime-boost approaches

One single injection of vaccine is seldom enough to induce protective immune responses. The immune system often needs multiple immunizations (e.g. booster doses), to prime strong immune responses. Repeated immunization of DNA, homologous boosting, does induce relatively inefficient immune activation in humans. Instead a more potent immune activation may be obtained using viral vectors. Pre-existing immunity to the viral vector can however be a problem using homologous boosting regimen. The pre-existing immunity could possibly clear the viral vector before the vaccine boosts the immune response. This could however be overcome by using a heterologous prime-boost strategy of one viral vector for priming and another for boosting (218). One combination that has been shown to work well is the combination of DNA prime and viral vector boost. This has been shown to be promising both in HCV and HIV vaccine studies (221, 268, 269). The DNA vaccine is easy to produce, store and transport, and associated with low-costs. However, combining plasmid DNA with viral vectors in a prime-boost regimen results in loosing some of the advantages with DNA vaccines. Instead DNA could be used with delivery techniques and addition of adjuvants to induce the immune response required.

3.5 ADJUVANTS

Adjuvants, from the Latin word adjuvare meaning “to help”, are almost always used in vaccines compositions with the aspect to help induce or shift the immune response. One of the most widely used adjuvants is Alum (aluminum potassium sulfate) (270). This adjuvant is used together with

proteins or subunit vaccines where it is mixed to an emulsion that are injected. How Alum works is not fully understood but it has been suggested that Alum stimulate $CD4^+$ T cells and humoral immune response by interacting with membrane lipids on dendritic cells (271). Another adjuvant used is the MF59 that is also mixed to an emulsion with the vaccine. This adjuvant has been used to induce antibody responses for HCV vaccines and other vaccines like HIV and influenza (217).

Toll-like receptor ligands can also be used as adjuvants and one TLR ligand that is indirectly used in all DNA vaccines is unmetylated cytidine-phosphate-guanosine (CpG) motifs. The unmetylated CpG motifs are detected by the TLR-9 as a defense against bacteria. Plasmid DNA is produced in bacteria and often contains unmetylated CpG motifs (272-274). Synthetic CpG motifs as oligodeoxynucleotides have also been used as adjuvants with the same mechanism of activation. Other TLR targets are TLR-5 and the bacterial compound flagelin and TLR-3 that is activated by double stranded RNA and also synthetic double stranded RNA e.g. polyinosine-deoxycytidylic acid (poly(I:C)) (275, 276). The triggering of TLRs and thereby the innate immune system is important for activation of type I IFN and the adaptive immune response.

3.5.1 Cytokines as adjuvant

Cytokines can be used as adjuvants to enhance the immune response to vaccine antigens. The use of cytokines in vaccine compositions can assist in priming a preferred type of immune response or to activate a specific cell type. Cytokines can be administrated as a plasmid DNA and/or as a recombinant protein. The addition of cytokine may improve the T cell activation and/or the recruitment of antigen presenting cells, both favoring an enhanced immune activation. There are in particular three cytokines of interest for HCV vaccines, IL-2, IL-12 and IL-21. The cytokine IL-2 is of importance for immunological memory and clonal expansion of T cells. In DNA vaccines IL-2 has been shown enhance both humoral and cellular immune responses (277, 278). The IL-12 with the capacity of promoting a Th1 type of immune response has been of interest as an adjuvant. The co-expression of IL-12 has been shown to improve the immune response of DNA vaccines (255) (Levander et al., submitted manuscript). Notably, the level of IL-21 is elevated in patients that resolve acute HCV infection (104). This in combination with the capacity to induce proliferation and effector function of T cells makes IL-21 a highly interesting adjuvant candidate (279-281). Hence, the immunogenicity of DNA vaccines can be improved when used in combination with cytokines. However, one need to choose the cytokine to be included carefully based on which immune response that one want to raise (282). The half-life of cytokines is in general short and if included in a treatment regimen, one may need to prolong the half-life, as pegylation was necessary to prolong the half-life of IFNα (165, 184). Delivery of cytokine genes as DNA plasmids result in continuous expression of the cytokine gene at least as long time as the expression of the vaccine gene.

3.5.2 Immune stimulatory gene sequences as adjuvant

Numerous antigens have been evaluated for their possibilities to enhance immune activations over the years. One antigen that has been tested for adjuvant properties is the hepatitis B core antigen (HBcAg). The hepatitis B core antigen is primary presented by B cells, it binds single stranded RNA, and have been shown to be a strong inducer of TLR-7 (283). In paper III we have shown that human HBcAg act as an adjuvant to enhance the overall immune response to HCV NS3/4A. Similarly, the stork HBcAg has been shown to enhance the immune response of both HCV NS3/4A and NS5A. The major advantage of using stork HBcAg is that it will not cross react with human HBcAg and can

therefore be used as an adjuvant in patients previously exposed to HBV and/or infected with HBV (**paper IV**, Levander et al., submitted manuscript).

4 TCR GENE THERAPY

Activation or re-activation of T cells by vaccination is not the only strategy to activate immune responses. In addition one can also manipulate the immune system, for example by using gene therapy one can manipulate the T cell receptor (TCR) expression on effector T cells (CTLs). A strategy that has been shown promising is manipulation of peripheral blood mononuclear cells (PBMCs) *ex vivo*. Here, PBMCs from a patient or a matched donor are either genetically manipulated or clonally selected (284, 285). The selected or generated antigen-specific populations of cells are then expanded and re-infused into the patient.

Therapy based on TCR gene therapy has been used with success to treat melanoma and viral infections such as CMV and EBV (286, 287). This treatment for viral infections targeting the liver has been evaluated and tested clinically for HBV with promising results (288, 289). Treatment of HCV with this type of therapy has not been tested clinically but is under development (**paper II**) (290, 291).

Using the patients own T cells is an advantage because there will not be any problem regarding graft versus host rejection, which could cause unwanted effects when using donated PBMCs. Also, the T cells to be used for infusion are expanded outside the tolerogenic liver environment. Hence, the modified T cells will have cytotoxic activity against the target cells already *ex vivo* and do not need to acquire these functions *in vivo*. The generated/selected T cells can be infused in large quantities with the same specificity. Prior to infusion of the redirected T cells one may eliminate potential molecules or cells that suppress T cells (e.g. regulatory T cells) (292).

There are of course some limitations with this type of therapy approach such as a high cost of treatment (e.g. personalized treatment), and the preparation of the redirected T cells are time consuming. Even though this treatment strategy is associated with some limitations it could however be suitable for HCV-infected patients unable of using todays treatment (IFNα, ribavirin and/or DAAs) due to side effects, resistance or for other reasons (e.g. patient groups like children, pregnant women, patients with renal failure, liver transplanted patients or already treatment experienced patients where it is still discussed whether they can be treated with the todays treatment or not).

5 AIM OF THE STUDY

The over all aim was to develop and evaluate new therapeutic interventions for chronic hepatitis C virus infection.

Specific aims in individual papers I-IV:

Paper I

- ✓ To develop and evaluate hepatitis C virus (HCV) non-structural (NS) 5A DNA vectors as potential vaccine candidates for chronic HCV infection.

Paper II

- ✓ To generate and evaluate HCV NS5A-specific T cell receptors (TCRs) for their anti-HCV activity.
- ✓ To characterize TCR expression on human T cells and compare antigen specificity and functional avidity.

Paper III

- ✓ To investigate if heterologous T cells can help restore function in dysfunctional HCV NS3/4A-specific T cells during therapeutic vaccination.

Paper IV

- ✓ Characterization of T cell responses to HCV NS3/4A and NS5A to better understand the immune modulatory role of NS5A during immune priming and effector functions.

6 COMMENTS ON MATERIALS AND METHODS

6.1 ANIMALS

In this thesis different mouse strains have been utilized. All mice used for immunological studies had a H-2^b background. Wild-type C57BL/6J mice were used to analyze naïve vaccine immune responses. Different stable transgenic (Tg) mouse strains (e.g. NS5A-Tg, NS3/4A-Tg and NS3/4A/5A-Tg) were used that mimic an individual with chronic HCV infection with continuous expression of the viral proteins. We have shown that the HCV-transgenic mice have dysfunctional T cells to HCV. Similarly, patients with chronic HCV infection have dysfunctional T cells. Our transgenic mice are valuable for analyzing vaccine-primed immune activation in an individual with dysfunctional T cell response. Also, mouse strains with different alterations in the immune system (e.g. IFNγR2$^{-/-}$, CD4$^{-/-}$ and CD8$^{-/-}$) have been used to study their effects on priming and effector functions. Mice expressing the human MHC class I (HLA-A2.1) instead of the mouse (H-2^b) have been used to study human T cell responses in a mouse (HHD$^+$ H-2D$^{b-/-}$ β2m$^{-/-}$) (293).

Tabel 1. List of mouse strains used in this thesis.

Name of strain	Background	Haplotype
Wild-type	C57BL/6J	H-2^b
NS3/4A-Tg	C57BL/6J	H-2^b
NS5A-Tg	C57BL/6J	H-2^b
NS3/4a/5A-Tg	C57BL/6J	H-2^b
IFNγR2	C57BL/6J	H-2^b
CD4$^{-/-}$	C57BL/6J	H-2^b
CD8$^{-/-}$	C57BL/6J	H-2^b
HHD (HHD$^+$, H2D$^{b-/-}$, β2m$^{-/-}$)	C57BL/6J	H-2^b
NS3/4A-HHD	C57BL/6J	H-2^b
NS5A-HHD	C57BL/6J	H-2^b

Female mice were housed 5-10 mice per cage, fed with a commercial diet and were at the age of 6-10 weeks at the start of the experiment. Mice were housed and bred at Karolinska Institutet, Division of Comparative Medicine, Clinical Research Center, Karolinska University Hospital, Huddinge, Sweden or purchased from Charles River Laboratories (Sulzfeld, Germany) and the Jackson Laboratory (Bar Harbor, ME). An ethical committee for animal research at Karolinska Institutet approved all handling and procedure involving animals. The studies has been planed by taking the Three Rs into account (Replacement, Reduction and Refinement) where we have tried to find alternative methods to reduce and minimize suffering. We have, when possible, followed the same mouse over time by using the *in vivo* imaging system, which has reduced the number of animals used.

6.2 PEPTIDE ANTIGENS

Numerous peptides have been used to study T cell responses to the vaccine antigens. For NS5A there was no identified T cell epitope in C57BL/6J mice. We therefore performed an epitope mapping of the NS5A protein to identify T cell epitopes. By using 87 20-mer peptides with 15 amino acids overlap, the two NS5A cytotoxic T lymphocyte epitopes could be identified using an IFNγ ELISpot and their function could be verified by a cytotoxicity assay e.g. ^{51}Cr release assay. (**paper I**)

Mice expressing the human HLA-A2 molecule have been utilized to characterize NS5A-specific immune responses after vaccination as well as for generation of NS5A-specific TCRs (**paper II and IV**). We have monitored immune responses to four identified human HLA-A2 epitopes. Our results have revealed that immune responses were only mounted to one of the four epitopes after vaccination. The reason for this could be differences in epitope sequences between the original genotype (genotype 1) and our vaccine (genotype 1b). All four HLA-A2 epitopes have shown to be present in patients with HCV infection.

6.3 PLASMID VECTORS AND VACCINE OPTIMAZION

Plasmids encoding the HCV NS3/4A and NS5A genes as well as HBcAg and SHBcAg have been used within this thesis. The constructs including the above mentioned genes have been design as single gene constructs and fusion gene constructs. The NS3/4A and NS5A proteins have important functions in the HCV life cycle and have a limited variability (44). Since both proteins are shown to be targets during HCV clearance they are also interesting as targets for HCV vaccines (103, 294). To enhance the immune response to NS3/4A and NS5A we included human and stork HBcAg as genetic adjuvants. Vaccine constructs with different modifications including addition of full-length and fragmented human/stork HBcAg antigens were generated. Full-length and fragmented constructs contain either NS3/4A proteolytic cleavage sites or self-cleaving peptide sequences to allow cleavage of the proteins into indicated parts (**Figure 10**) (295, 296). The different layout of the vaccine constructs will allow for different processing and degradation of the translated proteins, which will possibly affect the *in vivo* immune activation. This allows us to select the vaccine construct that most efficiently activates an HCV-specific immune response. The major drawback of using human HBcAg, as an adjuvant for the HCV vaccine is the pre-existing immunity to HBcAg in individuals exposed to, or infected with HBV. We solved this by instead using stork HBcAg, which has low cross-reactivity with human HBcAg and still is highly immunogenic.

The codon optimized (co) NS3/4A gene is generated from a genotype 1a and has been described previously (234). In addition, nine fusion constructs of NS3/4A and human HBcAg were synthesized

and used. The wild-type (wt) NS5A gene is derived from a genotype 1b isolate and described previously (297). Codon optimization of the wtNS5A gene and four NS5A and SHBcAg fusion constructs were synthesized and evaluated for immunogenicity. The plasmids were synthesized and generated by Retrogen (SanDiego, CA). Codon optimization is used to increase the gene expression by changing the codons in the vaccine to the most frequently used codons in humans.

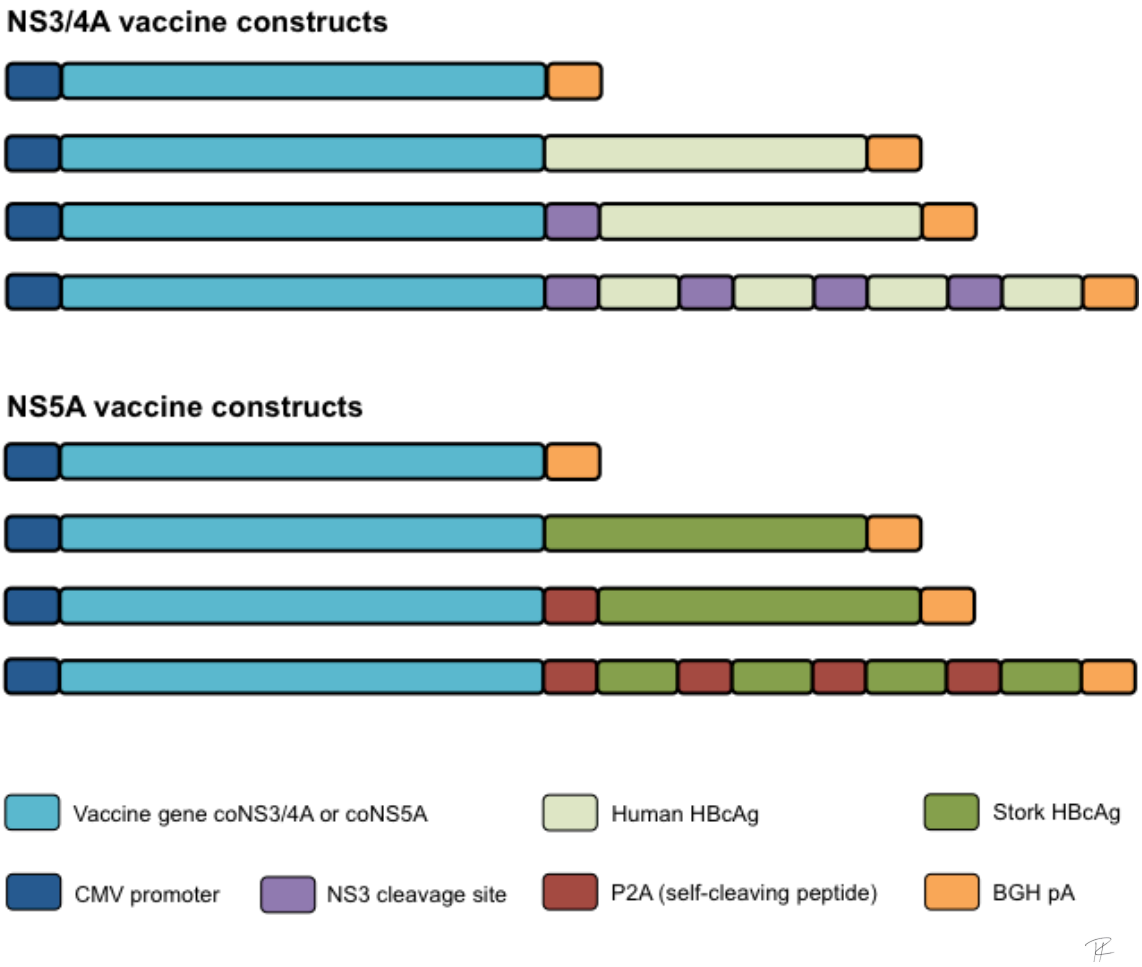

Figure 10. Design of HCV vaccine constructs used within this thesis.

6.4 IMMUNIZATION AND DELIVERY

As discussed earlier (*Delivery*) the uptake of plasmid DNA by eukaryotic cells is one parameter that needs to be improved to make DNA vaccines realistic for humans. One way is to optimize the immunization strategy using other injection techniques than regular intramuscular injection. *In vivo* intracellular injection device (IVIN) uses high pressure to localize the injected DNA solution to a defined area and thereby force the DNA to be taken up by the cells. Another strategy to enhance the uptake of DNA is to electroporate the injected area using an electroporation device. Within this thesis we have utilized two different devices, the MedPulser DNA delivery system (Inovio Pharmaceuticals, Blue Bell, PA) and the Cliniporator^2 (IGEA, Capri, Italy). The MedPulser DNA delivery system is a pre-set system with no possibility to change the pulse pattern (two 60 ms pulses of 246 V/cm). The

Cliniporator[2] on the other hand is more flexible and allow use of different pulse parameters. We found that one short high-voltage pulse followed by one long low-voltage pulse (one 1 ms 600 V/cm pulse and one 400 ms 60 V/cm pulse) was most efficient for uptake of our DNA vaccines. Our experience is that electroporation of the immunized tissue should be done directly after the immunization. One way to further optimize the vaccine delivery and uptake would be to combine the IVIN with electroporation in a one step procedure.

In the herein described studies mice were immunized with NS3/4A- or NS5A-based DNA vaccines or protein/s. In all experiments the number of mice were between 5-10 per group. Protein-based immunization was carried out using s.c. injection (50 μg) in the base of the tail. DNA-based immunizations were performed in the right tibialis anterior/cranialis (TA/TC) muscle using a regular intramuscular injection (i.m.) or by using the *in vivo* intracellular injection device (IVIN) in combination with *in vivo* electroporation. Vaccine-doses ranged between 5-300 μg, and mice were immunized one to three times at monthly intervals.

6.5 GENERATION OF TRANSIENTLY TRANSGENIC MICE

Transient expression of HCV and Firefly luciferase (FLuc) genes in the liver of mice were performed by a hydrodynamic injection two weeks after last immunization. Injection of 1,8 mL DNA solution (100 μg) in the tail vein within 10 second generates a high pressure in the liver resulting in uptake of DNA and subsequent expression of the plasmid encoding gene (*described in Model systems for HCV*). The protein expression can be detected from 4 hours up to 2 weeks after the hydrodynamic injection and with the maximum expression between 48-72 hours. We evaluated our vaccines in the transiently transgenic mouse model since it allowed us to study priming of HCV-specific immune responses and *in vivo* killing of HCV-expressing liver cells (**paper IV**). A functional vaccine should be able to induce T cell responses that can eradicate HCV-expressing hepatocytes. Weak or undetectable levels of intrahepatic HCV-protein are a result of a complete eradication of HCV transfected hepatocytes. Hence, the model allows for detection of HCV-protein expression in liver and in liver biopsies determined by *in vivo* imaging, immunohistochemistry and western blot. This method is of importance to determine the functional activity of the primed T cells. However, this model does not provide any information about which epitopes the T cells are targeting. The *in vivo* imaging system make it possible to visualize the expression of the plasmid in the liver. It also allow us to follow the same mouse over time and is of course also valuable in the terms of the three Rs. Western blot analysis is sensitive and the easiest way of comparing HCV-protein expression in different liver samples. Immunohistochemistry is valuable in the way of visualizing the histology and distribution of the HCV-protein in the liver and also to determine inflammation and infiltration of immune cells in the liver. One limitation of using western blot and immunohistochemistry is that the samples originate from a biopsy of the liver and does not represent the whole liver. The described methods for detection of HCV-protein expression have been used in combination to make it easier to interpret the result obtained from this model.

6.6 TRANSGENIC MICE

The transgenic mice used in this thesis are transgenic for the NS3/4A or NS5A genes or a combination thereof. The NS3/4A-Tg and NS5A-Tg mice differ in some aspects important for the interpretation of the results. The NS3/4A-Tg mouse strain is under the control of the mouse major urinary protein (MUP) promoter where the liver-specific NS3/4A-expression is turned on first two weeks after birth

(144). The NS5A-Tg mouse strain is expressed under the control of the mouse albumin (mAlb) promoter (297). The mAlb promoter generates a high liver-specific expression of the NS5A protein and the expression is active during neonatal development. Hence, these promoters have different characteristics in regards to expression levels and initiation of expression under neonatal development or after birth. This could result in that the NS5A-Tg mice have a more profound immunological tolerance compared to the NS3/4A-Tg mice. Thus, the NS3/4A-Tg mice are therefore possibly better in mimicking the situation during chronic HCV infection due to the onset of the HCV-expression after birth.

6.7 IMMUNOLOGICAL METHODS

6.7.1 Enzyme-Linked ImmunoSpot (ELISpot) assay

Clearance of HCV infection has been associated with IFNγ secretion. In addition, the IL-2 cytokine is of importance for maintaining the T cell response and memory T cells (110, 298). We have used ELISpot assay to quantify the IFNγ and IL-2 secretion after immunization. This method detects secretion of cytokines or other secreted components triggered after re-stimulation with MHC class I or II peptides *ex vivo*. The secreted cytokine from cells are captured by anti-cytokine antibodies coated on the bottom of a plate, after removal of the cells the secreted cytokine can be detected via biotinylated antibody and visualized by avidin-HRP, resulting in spots for each cytokine producing cell. This method has been well accepted for immune studies in human and animals due to its high reproducibility and sensitive. A limitation with this method is that it only analyzes a singe cytokine at a time. However, today the use of fluorespot assay makes it possible to determine multiple cytokines in the same assay. Another aspect is the use of a mixed population of cells (e.g. spleen cells) in the assay setup. A cell sorting approach may solve this limitation. On the other hand, the ELISpot assay enable the possibility to test several antigens at the same time, which is important for analyzing activated immune responses after immunization.

6.7.2 Detection of HCV-specific CTLs e.g. ^{51}Cr release assay

The ^{51}Cr release assay is one standard method to detect the functionality of cytotoxic T cells *ex vivo*. Target cells used in this method are RMA-S cells that are transporter associated with antigen processing (TAP) deficient and thereby unable to present endogenous peptides on the MHC class I. The cells are instead pulsed with peptides that correspond to identified CTL epitopes resulting in that the target cells present the peptide. The target cells are then incubated with ^{51}Cr, which is passively taken up by the target cells. After washes, the target cells can be added to the effector cells. Cytolytic killing of the target cells results in release of ^{51}Cr in the medium that can be detected using a β/γ-counter. This method provides important information about the function of activated CTLs. Data from this method is a valuable tool for evaluating the efficiency and functionality of vaccines. However, this method also has limitations, which is that the effector cells are re-stimulated with peptide or with cells stably expressing HCV proteins during five days *in vitro*. This re-stimulation outside the tolerogenic environment in the HCV-transgenic mice could result in high frequencies of killing that perhaps could be misleading and the actual *in vivo* effects of the killing is not so pronounced. This should be kept in mind when using this method and for evaluation of *in vivo* function of the primed immune response in the stable transgenic mice. Another method that can be used instead or in parallel is *in vivo* challenge with tumor cells expressing the vaccine-antigen. This model can be used to

monitor protection against tumor growth to investigate the functionality of vaccine-primed immune responses.

6.7.3 Polyfunctional T cells and pentamer

It has been shown that HCV-specific polyfunctional T cells are important during the clearance and resolution of HCV infection (105). The use of flow cytometry makes it possible to analyze cytokines and other molecules, both intracellular and at the cell surface. Analyzing polyfunctional T cells involves re-stimulation outside a tolerogenic milieu *in vitro* that could as in ^{51}Cr release assay result in a different response compared to the *in vivo* situation. However, the re-stimulation is rather short (6-12 hours). The detection of cells that binds a certain MHC class I epitope presented on a constructed MHC class I molecule can be done using reagents such as pentamer, tetramer or dimer X (dimer), where pentamers are the most stable molecules. This method is usually performed directly *ex vivo* and by that detecting HCV-specific cells as fast as possible outside the tolerogenic environment without any re-stimulation. Hence, this assay determines the frequency of HCV-specific T cells after immunization and could preferentially be used in combination with the ELISpot assay. Pentamer staining and polyfunctional T cell analysis provide detailed information about cell types and molecules expressed during the infection or immune activation.

7 RESULTS

7.1 A SYNTETIC CODON-OPTIMIZED HEPTATITIS C VIRUS NONSTRUCTURAL 5A DNA VACCINE PRIMES POLYFUNCTIONAL CD8$^+$ T CELL RESPONSES IN WILD-TYPE AND NS5A-TRANSGENIC MICE [PAPER I]

In the first paper we characterized and evaluated the HCV NS5A protein as a potential vaccine candidate. The NS5A protein has vital functions during HCV replication and for assembly of new virus particles. Moreover it has been shown to interact with the host cell signaling and interfere with both the innate and adaptive immune system. To characterize the intrinsic immunogenicity of the HCV NS5A we used NS5A-DNA constructs expressing full-length wild-type (wt) NS5A, full-length codon optimized (co) NS5A and eight truncated wild-type NS5A constructs.

To study the NS5A-specific cellular immune response following immunization with NS5A-DNA, we needed to identify NS5A CTL epitopes. A total of 87 20-mer peptides (15aa overlap) were generated to map NS5A CTL epitopes within the full-length NS5A genotype 1b protein. Identified peptides were shown to induce strong IFNγ production determined by ELISpot assay, they showed a high avidity in stabilization assay and was able to induce CTLs that specifically eradicated target cells in a ^{51}Cr release assay. Two epitopes were identified corresponding to the same sequence; one bound H-2D^b (VILDSFDPL, aa 2251-2259) and the other H-2K^b (ILDSFDPL, aa 2252-2259).

Immunization of wild-type mice with NS5A-DNA induced high levels of IFNγ and IL-2 secretion as well as T cells with cytolytic activity (**Figure 11**, and data not shown). Detection of IFNγ and IL-2 secretion and cytolytic activity could also be detected in NS5A-Tg mice however at a lower magnitude (**Figure 11**, and data not shown). Our results show that the NS5A-Tg mice are immunologically tolerant to the NS5A protein, which mimics the situation in an HCV infected patient with dysfunctional immune responses to HCV.

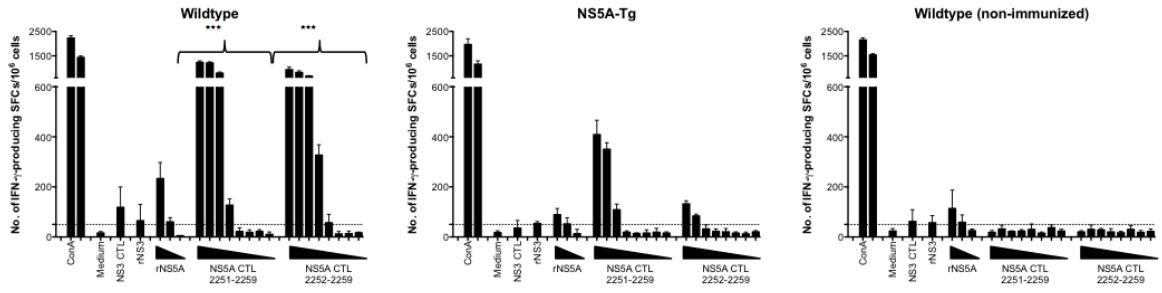

Figure 11. Quantification of NS5A-specific IFNγ-producing T cells per 10^6 splenocytes determined by an ELISpot assay. Mice were immunized i.m. once with 50 μg coNS5A-pVAX1 followed by *in vivo* electroporation of wild-type- and NS5A-Tg C57BL/6J mice.

The NS5A-based vaccine was further evaluated in a tumor challenge model to investigate the potential of primed T cells to eradicate tumors expressing NS5A. The wtNS5A- and coNS5A-DNA vaccines primed T cells that efficiently could inhibit *in vivo* tumor growth in wild-type and immuno-tolerant NS5A-Tg mice. This highlights that the NS5A-DNA vaccine has the ability to prime strong T cell responses also in the presence of chronic HCV-protein expression.

Additionally, the NS5A-DNA vaccine induced polyfunctional T cells that expressed TNFα, IL-2, IFNγ and CD107a in wild-type mice. Lower expression levels were seen in NS5A-Tg mice, albeit present. Importantly, the NS5A-DNA vaccine was able to activate a polyfunctional T cell response, which has been shown to be of importance in resolution of HCV infection.

7.2 TCR-REDIRECTED HUMAN T CELLS INHIBIT HEPATITIS C VIRUS REPLICATION: HEPATOTOXIC POTENTIAL IS LINKED TO ANTIGEN SPECIFICITY AND FUNCTIONAL AVIDITY [PAPER II]

In the second paper we generated antigen-specific polyfunctional T cells via TCR redirection. These TCRs were created by NS5A-DNA immunization in HLA-A2 transgenic mice, re-stimulated *in vitro* ($NS5A_{1992-2000}$) and thereafter purified $CD8^+$ T cells were fused with Thymoma cells (BW5147). The sequence of the TCR α- and β-chain were codon optimized, linked together with a F2A self-cleaving sequence and inserted in a retroviral vector used for gene transfer of the TCRs. The NS5A TCR transfected human T cells had a lower functional avidity and needed a higher concentration of peptide to be stimulated compared to the high avidity NS3 TCRs ($NS3_{1073-1081}$) generated previously. Transfection of NS3 and NS5A TCRs into T cells from healthy blood donors and HCV infected patients resulted in IFNγ production. This was evaluated after stimulation with NS3 and NS5A peptides and with non-transfected T cells as controls.

Polyfunctional T cells have been shown to be associated with control and clearance of HCV infection. We therefore tested the polyfunctionality of the T cells transfected with the NS3 and NS5A TCRs. T cells transfected with NS3 TCR were considered polyfunctional with expression of TNFα, IL-2 and IFNγ in both healthy blood donors and HCV infected patients. It was shown that 50 % of the T cells expressing the NS3 TCR were triple or double positive and the other 50 % were single positive for one of the tested cytokines. On the other hand, the majority of NS5A TCR transfected T cells expressed a single cytokine.

We also tested the cytolytic activity of the TCRs by analyzing the CD107a, which is a marker for recent lytic activity by perforin-granzyme mediated killing. This resulted in different profiles for the NS3 and NS5A TCRs. The CD107a was up regulated on the NS3 TCR transfected T cells but not on NS5A TCRs. Interestingly, even though the NS3 and NS5A effectively inhibit HCV replication in human hepatoma cells they do so with different mechanisms.

7.3 HETEROLOGOUS T CELLS CAN HELP RESTORE FUNCTION IN DYSFUNCTIONAL HEPATITIS C VIRUS NONSTRUCTURAL 3/4A-SPECIFIC T CELLS DURING THERAPEUTIC VACCINATION [PAPER III]

The chronic HCV infection is associated with impaired function of HCV-specific T cells expressing exhaustion markers on the cell surface. It has been shown that robust and broad T cell responses are essential to resolve chronic HCV infection and we therefore investigated if recruitment of healthy heterologous T cells could help in priming more potent NS3/4A-specific T cells.

First we tested if depletion of regulatory T cells or inhibitory molecules could restore the T cells function in immune-tolerant NS3/4A-Tg mice with impaired T cell responses compare to wild-type mice. The result showed that depletion of regulatory T cells, inhibitory molecules or multiple immunizations could restore the T cell function in NS3/4A-Tg mice. This indicates that NS3/4A-Tg

mice have impaired T cells caused by immune modulatory effects similar to those seen in patients with chronic HCV infection. Importantly, the T cells are not depleted by clonal deletion.

The hepatitis B core antigen (HBcAg) is known as a strong activator of the immune system via TLR-7. We therefore tested if this highly immunogenic protein could help to recruit healthy heterologous T cells and improve the T cell response when co-expressed with NS3/4A. The vaccine constructs including NS3/4A were modified by introduction of the adjuvant gene HBcAg. Fusion constructs including NS3/4A and human HBcAg were designed. Introduction of NS3/4A cleavage sites in the proteins resulted in fragmented versions of the HBcAg antigen. The introduction of cleavage sites in the NS3/4A-HBcAg vaccine constructs aimed to promote a different post-translational processing of the expressed proteins.

Results obtained using the different constructs revealed that human HBcAg weakly improved the T cell responses to NS3/4A in wild-type mice. Interestingly, the addition of human HBcAg significantly improved the NS3/4A-specific T cell response in the NS3/4A-Tg mice. Furthermore, we established a new NS3/4A-Tg mouse that expressed the human HLA-A2 instead of the mouse H-2^b. This mouse was used to study HLA-A2 NS3/4A-specific T cell responses in the presence of intrahepatic NS3/4A-expression. Immunization revealed that NS3/4A-DNA vaccines containing human HBcAg could restore the NS3/4A-specific T cell response whereas vaccines without HBcAg failed.

7.4 FUNCTIONAL DIFFERENCES IN HEPATITIS C VIRUS NONSTRUCTURAL (NS) 3/4A- AND NS5A-SPECIFIC T CELL RESPONSES [PAPER IV]

The NS5A protein is one of the least characterized proteins of HCV. In this study we wanted to compare the T cell response to HCV NS3/4A and NS5A with the aim of improve our understanding of the immune modulatory role of NS5A during immune priming and effector functions. We therefore further developed our NS5A-DNA vaccine (from paper I) and compared the NS3/4A and NS5A proteins in regards to priming of HCV-specific T cell responses. Priming of NS3/4A and NS5A T cell responses were in this study shown to be dependent on different molecules and cells of the immune system.

In this study we could show that the NS5A-DNA vaccine required much higher doses compared to NS3/4A-DNA to induce HCV-specific immune responses analyzed by IFNγ and IL-2 production and quantification of HCV-specific $CD8^+$ T cells. We know from previous studies that NS3/4A-specific T cell responses are dependent on $CD8^+$ T cells but not $CD4^+$ T cells and that depletion of regulatory T cells expressing CD25 and GITR greatly improves the immune response in NS3/4A-Tg mice (234). Priming of NS5A-specific T cells were here shown to be dependent on both $CD4^+$ and $CD8^+$ T cells and surprisingly depletion of regulatory T cells expressing CD25 and GITR did not improve the priming of NS5A-specific T cells. The cytokines IL-12 and IL-21 are known to enhance the T cell responses primed by DNA vaccines (255, 277-281). The IL-12 has been tested in combination with NS3/4A with promising results but neither IL-12 nor IL-21 improved the NS5A-specific T cell response.

However, addition of stork hepatitis B core antigen (SHBcAg) could significantly enhance the immunogenicity of NS5A in wild-type- and NS5A-Tg mice. The vaccine based on NS5A-SHBcAg was in this study shown to be highly immunogenic in mice expressing human HLA-A2 instead of the mouse H-2^d (**Figure 12**).

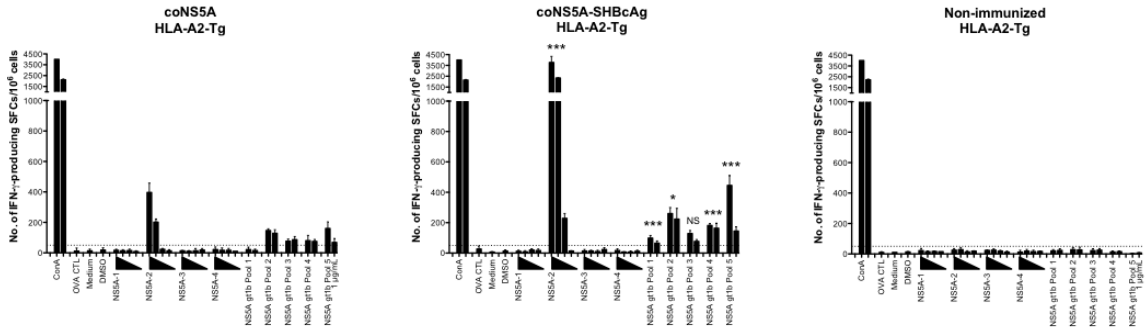

Figure 12. Quantification of NS5A-specific IFNγ-producing T cells after immunization of 50 μg NS5A-DNA vaccine with or without SHBcAg. Data presented as IFNγ-producing T cells per 10^6 splenocytes determined by an ELISpot assay.

In paper III we showed that there are differences in the functional properties between NS3 and NS5A TCR transfected T cells. In this study we compared the production of IFNγ and the inhibition of HCV replicon RNA replication. The NS3 TCR transfected T cells produced high levels of IFNγ and efficiently inhibited HCV replicon RNA replication whereas the NS5A TCR transfected T cells produced lower levels of IFNγ and less efficiently inhibited HCV replicon RNA replication.

In addition, differences between NS3/4A and NS5A were also found when analyzing the ability of primed T cells to clear intrahepatic NS3/4A- and NS5A-expression in transiently transgenic mice. Clearance of NS3/4A-expressing hepatocytes has been shown to be dependent on $CD8^+$ T cells and IFNγR2 but not $CD4^+$ T cells (66). Clearance of NS5A-expressing hepatocytes required both $CD4^+$ and $CD8^+$ T cells, but not IFNγR2, which is a distinct difference from NS3-specific T cells.

8 DISCUSSION

The hepatitis C virus is a major cause of liver disease and a major global health problem. It is estimated that around 130-150 million people are chronically infected, which corresponds to around 2 % of the world population. The infection becomes chronic in around 80 % of the cases and these patients may over time develop fibrosis with an increased risk of cirrhosis and hepatocellular carcinoma. There has been a revolution regarding treatment for HCV with numerous direct-acting antiviral drugs (DAAs). Even though the new treatment has a cure rate above 90 % there are still obstacles with the treatment. Firstly, the treatment is associated with high-costs, which result in limited or no use in resource-poor countries. It is estimated that only 10 % of all chronic HCV infected patients get any treatment (183). Secondly, there are still discussions whether patient groups like children, pregnant women, liver transplanted patients, patients with renal failure or already treatment experienced patients will be treated with the new antiviral compounds. Lastly, the treatment based on DAAs does not protect against re-infection. It is desirable with a future treatment that also protect against re-infection. To reduce HCV globally there is a need of treatment associated with lower costs. A treatment that activates the HCV-specific T cell response will most probably also be beneficial for patients with drug resistant virus, non-responder patients and patients that discontinued treatment due to side effects.

This thesis focuses on the HCV NS3/4A and NS5A proteins known to be important for the viral propagation. The NS3/4A protein has essential functions as protease and helicase in the viral life cycle. The NS5A protein is important for the viral replication and particle assembly as well as to interact with key players in the host cell signaling pathway. Furthermore, both NS3/4A and NS5A have been shown to interfere with the innate and adaptive immune system. Studies have demonstrated that NS3/4A and NS5A harbor T cell epitopes important for resolution of HCV infection. This, in combination with the fact that NS3/4A and NS5A also works as target for DAAs, makes them interesting as a vaccine candidates and targets for T cell based therapies.

Paper I: In the first study, we generated a HCV NS5A-based genetic vaccine of genotype 1b. The gene sequence was codon-optimized to maximize the expression level of the vaccine antigen in human cells. In addition, we also generated a wild-type (wt) construct and eight truncated constructs. First, we had to identify murine CTL epitopes within NS5A. The use of overlapping peptides resulted in identification of two functional epitopes in domain two of NS5A. These epitopes belonged to two different H-2^b alleles even though they corresponded to the same amino acid sequence. The identified epitopes induced IFNγ and IL-2 production and the primed specific CTLs had lytic activity.

The NS5A-based vaccines were tested in wild-type and immuno-tolerant (NS5A-Tg) mice with activation of IFNγ and IL-2 production and quantifiable numbers of NS5A-specific $CD8^+$ T cells. The responses were in general weaker, but detectable in the NS5A-Tg mice. Our results highlights that the NS5A-Tg mice have tolerized T cell responses to NS5A, and thereby serves as a relevant model for studying NS5A-specific immune responses. Importantly, our NS5A-DNA vaccine could prime NS5A-specific immune response in the NS5A immuno-tolerant transgenic mouse model.

Furthermore, the NS5A-DNA vaccine was also tested for functionality in a tumor challenge model. This model is based on a tumor cell line expressing NS5A. This model allowed us to investigate the protection against *in vivo* tumor growth after immunization. The NS5A-DNA vaccine was shown to protect wild-type mice and to a lower extent also immuno-tolerant NS5A-Tg mice. The tumor

challenge model was also used to characterize to which regions of the NS5A protein the NS5A-specific T cells were directed. Our results revealed that the C-terminal part of NS5A was shown to mediate the protection.

In addition to showing that the NS5A-DNA vaccine induced functional T cell responses, we analyzed whether the vaccine also could activate HCV-specific polyfunctional T cells. HCV-specific polyfunctional T cells have been shown to be of importance in the resolution of HCV infection (105). Our NS5A-DNA vaccine could prime polyfunctional T cells producing TNFα, IL-2, IFNγ and expressing CD107a in both wild-type and immuno-tolerant NS5A-Tg mice after a single immunization. All together, this shows that our NS5A-DNA vaccine is able to prime polyfunctional NS5A-specific T cells with lytic activity in the presence of chronic NS5A expression in the NS5A-Tg mice.

Paper II: In the second paper we used the NS5A-DNA vaccine (developed in paper I) to activate human NS5A-specific T cells in HLA-A2-Tg mice. The NS5A-specific T cells were used to generate NS5A-specific TCRs. The generated NS5A TCRs were compared with the previously generated NS3 TCRs for functional activity. The TCRs were specific for two T cell epitopes ($NS3_{1073-1081}$ and $NS5A_{1992-2000}$) known to correlate with resolution of HCV infection, (294, 299).

The NS3 and NS5A TCR-transfected T cells were shown to respond with IFNγ production upon stimulation with the respectively antigens. The NS5A TCRs did though need higher concentration of the antigen to respond. Differences between the TCRs were also seen in the polyfunctionality where the NS3 TCRs was associated with polyfunctional T cells with 50 % of the TCR-redirected T cells positive for two or three of the following cytokines; TNFα, IL-2 and IFNγ. The NS5A TCR-redirected T cells were instead single positive and mainly producing IFNγ or TNFα. The effector function of the TCRs were also different where the high avidity NS3 TCRs were shown to perform cytolytic effector functions while the low avidity NS5A TCRs instead performed non-cytolytic effector functions.

High avidity CTLs are sensitive to antigen-induced cell death but effective in clearance of virus infected cells. Control of HCV infection has been associated with selection of high avidity T cells, polyfunctionality and potent viral suppressive activity (300, 301). Low avidity CTLs on the other hand has been proposed to control disease progression during virus infection (302). Low avidity T cells have also been described to suppress viral replication in target cells without cytolytic effector function but instead with secretion of IFNγ and TNFα. This has been shown for HCV and HBV infection and mediated by virus-specific $CD8^+$ T cells (303, 304). The use of low-avidity TCRs in treatment of chronic HCV could be an advantage due to clearance of HCV infected cells without inducing any liver damage (303).

Mouse and human TCRs have shown high tolerability in humans as a therapy against cancer (292, 305, 306). A broad polyfunctional immune response to HCV is required for viral clearance (102, 103). According to this, using one or two TCRs to transfect human PBMCs may not be enough to obtain eradication of the viral infection. However the use of multiple TCRs may be an effective strategy to clear the infection. The strategy of redirecting HCV-specific T cells could be of interest for patients who have failed antiviral treatment or are infected with a drug resistant virus.

Paper III: In the third paper the aim was to enhance the immunogenicity of the previously developed NS3/4A-DNA vaccine by the addition of immune stimulatory gene-sequences. The immune system of patients with chronic HCV infection is exhausted and their T cells are commonly dysfunctional (100,

108, 111, 113, 115, 307). A majority of the HCV-specific T cells during chronic HCV infection are impaired. Hence, to successfully activate potent HCV-specific T cell responses in individuals with chronic HCV infection the immune priming must be highly immunogenic. This may be obtained by recruiting healthy heterologous T cells to the site of priming. This has previously been shown for non-responders to the hepatitis B vaccine. Immunization of the previous non-responders using both the hepatitis A and B vaccine induced a 95% seroconversion to HBcAg (308).

In this study we used NS3/4A-Tg mice known to have dysfunctional NS3/4A-specific T cells, which is similar to what have been shown for humans with chronic HCV infection. Immunization of the NS3/4A-Tg mice using the NS3/4A-DNA vaccine showed an impaired phenotype of the primed T cell responses. Interestingly, the NS3/4A-specific T cell responses could partly be restored by depletion of regulatory T cells or cells expressing inhibitory molecules revealing that the impaired T cell responses were due to dysfunctional T cells and not explained by clonal deletion.

We have in this and previous studies shown that the NS3/4A-DNA vaccine is priming potent NS3/4A-specific T cell responses in wild-type mice and weaker T cell responses in immuno-tolerated NS3/4A-Tg mice. The addition of human HBcAg gene-sequences to the NS3/4A-DNA vaccine resulted in weakly improved NS3/4A-specific T cell responses in wild-type mice. On the other hand, when human HBcAg and NS3/4A was co-expressed in the NS3/4A-Tg mice we found a significant improvement of the primed T cell response, evidenced by increased NS3/4A-specific IFNγ-producing T cells. The use of immune stimulatory gene-sequences to recruit healthy heterologous T cells to the site of immune activation was in this study effective and could reactivate the dysfunctional T cells and improve the overall immune response.

Since around two billion people have been exposed to hepatitis B, the use of human HBcAg may not be optimal as a vaccine adjuvant. Thus, one alternative is to use HBcAg from another species, such as the avian HBcAg. To avoid problems with pre-existing immunity we decided to replace the human HBcAg with avian HBcAg (e.g. stork HBcAg). Importantly, there are no or little cross reactivity between human and stork HBcAg.

Paper IV: In the fourth paper we combined the NS5A-DNA vaccine from paper I and the adjuvant evaluated in paper III. This new improved NS5A-DNA vaccine was in this study compared to the previously generated NS3/4A-DNA vaccine. We were interested in which factors that are required for NS5A-specific T cell priming. We also compared NS3 and NS5A TCR-redirected human T cells for anti-HCV activity *in vitro* as described in paper II, to address functional differences in NS5A and NS3/4A T cell priming.

We have previously shown that NS5A-DNA could induce NS5A-specific T cell responses in wild-type and NS5A-Tg mice. Next we analyzed if the immunogenicity of the NS5A could be further improved. We evaluated the vaccine dose, co-expression with cytokine genes, and the importance of $CD4^+$ and $CD8^+$ T cells for immune priming. We found that activation of NS5A-specific T cell responses was highly dose dependent. In addition we could show that NS5A-specific immune responses are dependent on both $CD4^+$ and $CD8^+$ T cells for priming. The immune response could not be adjuvanted by addition of IL-12 and/or IL-21. Notably is that the NS3/4A-DNA vaccine primed immune responses in the absence of $CD4^+$ T cells, and the immune response could be adjuvanted by IL-12 (234) (Levander et al., submitted manuscript). Depletion of regulatory T cells or inhibitory molecules did not improve the NS5A-specific immune activation. On the other hand, we have shown that NS3/4A-specific T cell responses can be improved by Treg depletion.

Interestingly, the addition of immune stimulatory gene-sequence (e.g. stork HBcAg) increased the overall immune response to both NS5A and NS3/4A. The adjuvant effect was weak in wild-type mice but more evident in the respective transgenic mouse linage. We have recently shown that NS3/4A-specific T cell responses can be adjuvanted by both human and stork HBcAg (Levander et al., submitted manuscript). Stork and human HBcAg show very limited cross reactivity, thus stork HBcAg may be used in patients with chronic HBV infection. Our results revealed that the NS5A-stork-HBcAg DNA vaccine was superior the NS5A-DNA vaccine alone in humanized mice (e.g. HLA-A2-Tg mice).

In addition, we compared the functional properties of the T cell responses primed after NS3/4A- and NS5A-DNA immunization. The NS3/4A-specific T cell response is known to be dependent on IFNγ, $CD8^+$ T cells but not $CD4^+$ T cells (66, 309). This is a distinct difference from primed NS5A-specific T cell responses that requires both $CD4^+$ and $CD8^+$ T cells for priming but not IFNγ. Moreover, we generated TCRs with specificity for NS3 and NS5A. We found functional differences in avidity, induction of IFNγ-production and the TCRs effector function. The NS3 TCRs (high avidity) with cytolytic activity was shown to inhibit *in vitro* HCV RNA replication. In contrary, the NS5A TCRs (low avidity) performed non-cytolytic activities that could inhibit HCV RNA replication. In conclusion, the HCV-specific immune response may be dependent on both these types of primed responses.

In summary, we have developed a NS5A-DNA vaccine with the capacity of inducing both IL-2 and IFNγ. Vaccine activated NS5A-specific $CD8^+$ T cells were polyfunctional and immune responses could be detected in both wild-type and NS5A immuno-tolerant mice. The NS5A-DNA vaccine was also analyzed in a tumor challenge model was primed NS5A-specific T cells protected against tumor growth. The NS5A-DNA vaccine was also used in the generation of NS5A-specific TCRs. The NS5A TCRs (low avidity) produced IFNγ and could inhibit HCV RNA replication. The inclusion of an immune stimulatory gene-sequences (human or stork HBcAg) in our vaccine compositions was shown to adjuvant both the NS3/4A- and NS5A-specific T cell response. Finally, a comparison of NS3/4A and NS5A reveled that the two vaccines can induce effector function but requires different factors for immune priming. The development of new alternative treatment strategies for HCV is needed to be able to treat patients in both high- and low-income countries.

9 GENERAL CONCLUSION

- ✓ Genetic immunization using HCV NS5A efficiently primed NS5A-specific T cell responses.
- ✓ Two HCV NS5A-transgenic mouse models (stable and transient) were developed and successfully used for evaluation of vaccine primed immune responses.
- ✓ Development of HCV-specific TCRs revealed correlation between antigen specificity and antiviral activity.
- ✓ Genetic immunization using HCV NS3/4A and NS5A revealed functional differences in the T cell response. Both priming and effector functions differed among the two antigens.
- ✓ Healthy heterologous T cells could restore dysfunctional HCV-specific immune responses.

10 POPULÄRVETENSKAPLIG SAMMANFATTNING

Hepatit C är ett virus som infekterar levern och sprids främst via kontaminerat blod. I Sverige introducerades screening för hepatit C under tidigt 1990-tal vilket har resulterat i att viruset idag huvudsakligen sprids mellan personer som injicerar narkotiska preparat. I låginkomstländer där kontroll av blod och blodprodukter samt sterilisering av medicinska instrument fortfarande är undermåliga sprids hepatit C fortfarande inom sjukvården. Förutom ovan nämnda smittvägar så är även tatueringar och piercing med oren utrustning möjliga smittkällor.

I Sverige finns idag ca 40 000 personer som lever med hepatit C virus (HCV) infektion och i hela världen mellan 130-150 miljoner vilket motsvarar omkring 2 % av den totala befolkningen. Hos 20 % av de som infekteras med HCV lyckas immunförsvaret själv läka ut infektionen och personen blir frisk. I de resterande 80 % av fallen blir infektionen kvarvarande (e.g. kroniska). I dessa fall misslyckas immunförsvaret med att kontrollera viruset vilket bland annat kan förklaras av att viruset är så föränderligt. Detta leder hela tiden till att nya virusvarianter framträder som immunförsvaret måste bekämpa.

Sedan identifieringen av HCV år 1989 har behandlingen utvecklats och de senaste åren har det skett en revolution gällande virushämmande läkemedel för HCV. De tidiga behandlingarna av HCV med IFNα resulterade i att 6 % blev friska, med kombinationen av IFNα och ribavirin 50 % och IFNα och ribavirin i kombination med de första virushämmande läkemedlen resulterade i ca 75 % blev botade. Interferon-α (IFNα) är en komponent i det naturliga försvaret som kroppen själv producerar som svar på en virusinfektion. IFNα kan även tillverkas och ges som läkemedel. Ribavirin är ett annat läkemedel som stör/förhindrar produktionen av nytt virus. Tyvärr så är behandlingar med IFNα och ribavirin associerade med flera besvärliga biverkningar och därför har målet varit att hitta alternativa behandlingar för att slippa använda dessa läkemedel. De nya virushämmande läkemedlen som finns idag har en utläkningsfrekvensen på över 90 % och är fria från IFNα och ribavirin samt ges i tablettform oftast en gång per dag.

Trots den nya behandlingens höga utläkningsfrekvens finns det flera nackdelar med behandlingen. Ett, den höga kostanden för läkemedlen som medför att användandet av dessa blir restriktivt till de svårast sjuka. Två, det finns patientgrupper som inte kan ta dessa läkemedel på grund av resistensutveckling, det vill säga patienter som utvecklar motståndskraft mot läkemedlet, och även de som inte tål behandlingen av olika orsaker. Dessutom är det ovisst huruvida barn, gravida och patienter oförmögna att svara på behandlingen ska behandlas med dessa läkemedel. Tre, de nya virushämmande läkemedlen ger inget skydd mot att smittas av HCV igen. I dagsläget får endast 10 % av alla världens HCV infekterade personer någon behandling och faktum är att de flesta infekterade finns i låginkomstländer vilket innebär att det finns ett stort behov av nya alternativa behandlingar som är mer kostnadseffektiva.

I nuläget finns inget vaccin och inte heller någon behandling som ger skydd mot återinfektion. Vi arbetar med att utveckla behandlingar som på ett effektivt sätt aktiverar immunförsvaret hos de som bär på HCV och därigenom kan utplåna HCV. Ett problem är att viruset hela tiden förändrar sin arvsmassa vilket göra att immunförsvaret ofta har problem att känna igen delar av viruset. Det är därför viktigt att rikta behandlingen mot de delar av viruset som förändras minst. Vi har baserat våra behandlingar till två virusstrukturer som förändrar sig mindre jämfört med andra regioner av HCV.

Dessa delar är de icke-strukturella proteinerna 3/4A och 5A. Dessa två proteiner är livsviktiga för att viruset ska kunna producera nya viruspartiklar.

Vår behandlingsstrategi baseras främst på genetiska vaccin där genen (e.g. arvsmassan) för det aktuella proteinet används som vaccin. Vaccinet som består av cirkulärt DNA innehåller vaccingenen och detta DNA kan odlas upp i stora mängder i bakterier. När vaccinet ges till människa kommer genen att utryckas till protein i cellerna. Cellerna kommer då att bryta ner proteinet och visa det för immunförsvaret på cellytan. Det här är samma sätt som celler annars använder för att presentera egna och främmande proteiner från virus och bakterier för immunsystemet. Det gör att immunförsvaret blir aktiverat på ett naturligt sätt, nämligen att både de celler som dödar infekterade celler (mördar T celler) och de celler som hjälper till att rikta immunförsvaret i rätt riktning (T hjälpar celler) aktiveras. Vårt vaccin har testats i möss där muskeln i ett av bakbenen injiceras med vaccin. Genen som kommer från viruset har modifierats så att den så lätt som möjligt uttrycks i djur och människa samt att den ges i kombination med en teknik som heter *in vivo* elektroporering. Tekniken syftar till att öppna upp porer i muskelcellerna och att dra in vaccin-DNAt i cellerna. Den ökade mängden vaccin-DNA som kommer in i cellerna genom *in vivo* elektroporering samt den optimeringen av vaccinet vi gjort ökar uttrycksnivåerna av protein i muskeln. Detta leder till ökad presentation av protein-delar för immunförsvaret och därmed får vi en starkare aktivering av immunförsvaret. Vi har även undersökt vilka komponenter som är viktiga och nödvändiga för att få en stark aktivering av immunförsvaret. Våra resultat visar att vaccinet som baseras på NS3/4A är beroende av T mördar celler för sin funktion medan NS5A vaccinet är beroende av både T mördar celler och T hjälpar celler. Genom att jämföra våra två vaccinkandidater har vi ökat vår förståelse om dessa proteiner samt deras förmåga att aktivera immunförsvaret. Vi har även lärt oss hur vi kan optimera dem för att få en starkare immunaktivering. Studier har visat att de T celler som känner igen viruset är utmattade och kan därför inte utplåna de infekterade cellerna. Vi har då försökt att utveckla våra vaccin genom att tillsätta vissa komponenter som ökar aktiveringen av immunförsvaret (adjuvant). Ett adjuvant som visade sig fungera för båda vaccinen var tillsatsen av en gen från ett annat virus (hepatit B virus). Genom att använda denna gen i kombination med våra vaccin-gener så har vi kunnat få en betydligt starkare aktivering av immunförsvaret vilket ökar möjligheterna till att immunförsvaret kan utplåna HCV.

Vår forskning syftar till att ta fram alternativa behandlingar mot HCV för att nå fram till så många patienter som möjligt. Ett genetiskt vaccin är billigt att producera och har en bra stabilitet vilket möjliggör transport och förvaring i rumstemperatur. Dessa egenskaper medför möjlighet att skapa ett behandlande vaccin som kan användas på bred front i så väl höginkomstländer respektive låginkomstländer.

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